UNIVERSIDADE ESTADUAL DE CAMPINAS
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ANA PAULA ALMEIDA AYRES

EFFECT OF NON-THERMAL ATMOSPHERIC PLASMA APPLICATION ON THE DENTINAL SURFACE AND ON THE ADHESION OF RESTORATIVE MATERIAL

EFEITO DA APLICAÇÃO DE PLASMA ATMOSFÉRICO NÃO TÉRMICO NA SUPERFÍCIE DENTINÁRIA E NA ADESÃO DO MATERIAL RESTAURADOR

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Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Dental Materials.

Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Materiais Dentários.

Orientador: Prof. Dr. Marcelo Giannini

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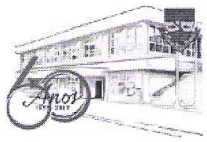

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EPÍGRAFE

"Não é sobre ter
Todas as pessoas do mundo pra si
É sobre saber que em algum lugar
Alguém zela por ti
É sobre cantar e poder escutar
Mais do que a própria voz
É sobre dançar na chuva de vida
Que cai sobre nós

É saber se sentir infinito
Num universo tão vasto e bonito
É saber sonhar
E, então, fazer valer a pena cada verso
Daquele poema sobre acreditar

Não é sobre chegar ao topo do mundo
E saber que venceu
É sobre escalar e sentir
Que o caminho te fortaleceu
É sobre ser abrigo
E também ter morada em outros corações
E assim ter amigos contigo
Em todas as situações

A gente não pode ter tudo
Qual seria a graça do mundo se fosse assim?
Por isso eu prefiro sorrisos
E os presentes que a vida trouxe
Para perto de mim

Não é sobre tudo que o seu dinheiro
É capaz de comprar
E sim sobre cada momento
Sorriso a se compartilhar
Também não é sobre correr
Contra o tempo pra ter sempre mais
Porque quando menos se espera
A vida já ficou pra trás

Segura teu filho no colo
Sorria e abraça teus pais
Enquanto estão aqui

Que a vida é trem-bala, parceiro
E a gente é só passageiro prestes a partir "

Trem Bala (Ana Vilela)

RESUMO

O objetivo neste estudo foi avaliar a influência da aplicação de plasma atmosférico não térmico na superfície dentinária, na interface dentina-resina e na resistência de união de um sistema adesivo universal, Scotchbond Universal (3M ESPE). Dois tempos de aplicação do plasma foram analisados: 10 e 30 segundos (seg), na técnica com condicionamento ácido prévio da dentina e na autocondicionante. No capítulo 1, a morfologia e a composição das superfícies tratadas com plasma foram determinadas com Microscopia de Força Atômica (n = 3) e Espectroscopia Confocal Raman (n = 5), respectivamente. A influência do plasma na atividade enzimática de metaloproteinases foi avaliada pelo método de Zimografia *in situ* (n = 3). Para determinação da resistência de união, utilizou-se o ensaio de microtração (n = 8) após o armazenamento dos espécimes por 24 horas e um ano. Dois métodos de envelhecimento *in vitro* foram utilizados: "exposição direta à água" e "pressão pulpar simulada", os quais produziram diferenças na efetividade de união e na distribuição dos padrões de fratura. Os espécimes armazenados por um ano na forma de "palitos" apresentaram maior prevalência de fratura coesiva em resina e queda da resistência de união apenas para os grupos tratados com plasma por 30-seg. Porém, os valores destes grupos não diferiram estatisticamente dos demais grupos avaliados após um ano. Quando as amostras foram armazenadas na forma de dentes restaurados, com câmara pulpar submetida à pressão de coluna d'água, não houve diferença entre os resultados de resistência de união imediatos e após um ano. Os grupos controles apresentaram médias significativamente mais baixas que os grupos tratados por plasma, tanto no modo convencional quanto no autocondicionante, com predominância de falha adesiva. Os resultados do Capítulo 1 indicam que a exposição de dentina hígida e desmineralizada ao plasma não produziu modificações morfológicas quanto à rugosidade da superfície, nem alterações nos espectros de 'carbonato' e 'colágeno tipo I'. Apenas o espectro do elemento 'fosfato' apresentou queda após aplicação de plasma por 10-seg. Na técnica adesiva convencional, as imagens zimográficas da dentina não tratada com plasma apresentaram maior fluorescência verde, o que é indicativo de alta atividade enzimática, principalmente na região de camada híbrida. No Capítulo 2, os mesmos grupos foram restaurados e as amostras armazenadas por dois anos apresentaram queda de resistência de união à microtração (n = 8), na técnica autocondicionante. Porém, os grupos tratados com plasma mantiveram os valores de resistência de união imediatos. O tratamento com plasma também aumentou a hidrofilia da dentina e produziu maiores valores de nanodureza (n = 3) e módulo de elasticidade (n = 3) da camada híbrida, em comparação com os grupos controles. Pode-se concluir que a aplicação de plasma atmosférico não térmico em dentina produziu melhores resultados no tempo de 30-seg, sem significantes alterações físico-químicas da superfície dentinária e com aumento considerável da sua hidrofilia. A respeito da interface dente-restauração, o tratamento produziu maiores médias de nanodureza e módulo de elasticidade na camada híbrida, sem aparentemente aumentar a atividade enzimática nessa região. Esses efeitos parecem ter contribuído para a longevidade adesiva.

Palavras-chave: Gases em plasma. Resistência à tração. Dentina.

ABSTRACT

This study aimed to evaluate the influence of non-thermal atmospheric plasma application onto the dentin surface, in the dentin-resin interface and in the bond strength of a universal adhesive system, Scotchbond Universal (3M ESPE). Two times of plasma application were analyzed: 10 and 30 seconds, in etch-and-rinse and self-etch adhesive techniques. In Chapter 1, the morphology and composition of plasma-treated surfaces were determined by Atomic Force Microscopy (n = 3) and Raman Confocal Spectroscopy (n = 5), respectively. Plasma influence in enzymatic activity of metalloproteinases was assessed by *in situ* Zimography. Microtensile test was used to determine the bond strength (n = 8) after specimen storage for 24 hours, one year and two years. Two aging methods were utilized: “direct water exposure” and “simulated pulpal pressure”, which produced differences in bonding effectiveness and in failure pattern distribution. One-year stored specimens in “beam” shape showed prevalence of cohesive within resin failure and the bond strength decreased only for 30 s plasma-treated groups. Although these values did not statistically differ from the other groups after one year. When the samples were stored as restored teeth, with pulp chamber submitted to water column pressure, there was no difference among immediate and one-year bond strength, indicating bonding durability. Control groups showed statistically lower mean values, in etch-and-rinse and self-etch approaches, with predominant adhesive failure. The results from Chapter 1 indicate that plasma application on intact and demineralized dentin did not produce morphologic modifications related to surface roughness, neither alterations in ‘carbonate’ and ‘type I collagen’ spectrums. Only the ‘phosphate’ spectrum decreased after plasma application for 10 s. In etch-and-rinse technique, zimography images of untreated dentin showed greater green fluorescence, which indicates high enzymatic activity, mainly in the hybrid layer. In Chapter 2, the same groups were restored and the samples stored for two years showed a decreased microtensile bond strength (n = 8), in self-etch technique. However, the groups that were plasma-treated kept the immediate bond strength values. Plasma treatment also increased the dentin hydrophilicity and produced higher nanohardness and Young’s modulus means of the hybrid layer, when compared to control groups. In conclusion, the application of non-thermal atmospheric plasma onto dentin produced better results in the time of 30 s, without significant physical-chemical alterations of dentinal surface, increasing dentin hydrophilicity considerably. Regarding tooth-restoration interface, the treatment produced higher nanohardness (n = 3) and Young’s modulus (n = 3) means in the hybrid layer, without apparently increase the enzymatic activity in this area. These effects seem to contribute to bonding longevity.

Key-words: Plasma gases. Tensile strength. Dentin.

SUMÁRIO

1 INTRODUÇÃO

O estado da arte dos adesivos contemporâneos tem apresentado resultados imediatos de adesão muito satisfatórios (Giannini *et al.*, 2015; Ástvaldstóttir *et al.*, 2015). Tempos cada vez maiores de avaliação clínica e envelhecimento *in vitro* são necessários para se detectar sinais de degradação e determinar a longevidade da união dentina-resina. Todavia, considerando-se a expectativa de que uma restauração adesiva dure o máximo possível em função e com manutenção de saúde dos tecidos adjacentes, medidas que melhorem a qualidade e prolonguem a durabilidade da interface dente-restauração continuam bem-vindas e devem ser estudadas. Para isso, um bom conhecimento das propriedades dos materiais restauradores utilizados, assim como das características dos tecidos dentais são essenciais para o desenvolvimento de estratégias restauradoras com sucesso a longo prazo (Beck *et al.*, 2015).

O tecido dentinário é constituído por aproximadamente 50% em volume de minerais, sendo o restante composto por colágeno tipo I e proteínas não colágenas (30% em volume) e água (20% em volume) (Tjärderhane *et al.,* 2009). Na técnica restauradora adesiva, a superfície dentinária tem a porção mineral totalmente removida pelo condicionamento com ácido fosfórico quando se utiliza a técnica convencional ou parcialmente removida pelos monômeros ácidos dos *primers* e/ou adesivos na técnica autocondicionante. A matriz colágena exposta é então infiltrada pelo sistema adesivo fluido, o qual polimeriza após a fotoativação. Principalmente com a técnica convencional, os monômeros dos adesivos não conseguem encapsular totalmente as fibrilas da rede de colágeno, deixando-as total ou parcialmente expostas, principalmente na base da camada híbrida, quando deveriam idealmente ficar protegidas com a polimerização da resina adesiva (Tjärderhane *et al.,* 2013). Essa falta de proteção e a presença de água residual e/ou advinda da polpa através dos túbulos dentinários, tornam a região de interface dente-restauração vulnerável às degradações hidrolítica e enzimática. Ambas são interligadas e tempo-dependentes, podendo levar à destruição da camada híbrida, diminuição de resistência de união e de propriedades mecânicas com o passar do tempo (Frassetto *et al.,* 2016).

Visando a superar os desafios da longevidade limitada, várias estratégias têm sido investigadas na Odontologia Adesiva, tais como: incorporação de inibidores de metaloproteinases em *primers* e/ou adesivos, aplicação de "*cross-linkers*", uso de EDTA para condicionamento da dentina, técnica de substituição da água entre as fibrilas da dentina condicionada utilizando etanol, agentes remineralizantes da camada híbrida, entre outras (Tjärderhane *et al.,* 2013). O tratamento da superfície dentinária utilizando plasma atmosférico não-térmico é uma abordagem ainda pouco explorada na Odontologia. Essa tecnologia produz espécies altamente reativas num meio gasoso, à temperatura fisiológica ou abaixo dela, responsáveis por aumentar a hidrofilia e reatividade do substrato. Acredita-se que dessa forma a efetividade de infiltração do adesivo seja melhorada, reduzindo os espaços vazios entre as fibrilas colágenas (Dong *et al.,* 2013; Chen *et al.,* 2013; Chen *et al.,* 2014) e permitindo assim uma desaceleração dos processos envolvidos na degradação da interface dentina-resina.

Esta inovação tem sido utilizada em diversas aplicações industriais como uma opção viável para modificar propriedades químicas da superfície de diferentes substratos e materiais (Becker *et al.,* 2006; Foest *et al.,* 2006). Desta forma espera-se observar similar efeito na superfície dentinária com o tratamento da *smear layer*, e redução da energia de superfície, o que permitiria melhor molhamento por parte do adesivo fluido. Uma revisão publicada recentemente resumiu os avanços do plasma na Odontologia Adesiva (Liu *et al.,* 2016), tais como: melhores resultados de resistência de união, aumento da hidrofilia da dentina, aumento da polimerização monomérica, melhor infiltração do adesivo na dentina, dentre outros. O efeito do tratamento com plasma depende de vários fatores como tempo de aplicação, gás utilizado, quantidade de energia e frequência dispensadas pelo aparelho (Chen *et al.,* 2014; Han *et al.,* 2014; Ritts *et al.,* 2010), assim como fatores relacionados ao substrato (Lehmann *et al.,* 2013; Chen *et al.,* 2013).

O presente estudo buscou avaliar se o tratamento com plasma atmosférico não-térmico promoveria alterações na superfície dentinária e na efetividade de união. Levando em consideração a versatilidade dos sistemas adesivos "universais" ou "multimode", o ensaio de resistência de união por microtração foi realizado nas técnicas adesivas convencional e autocondicionante, associada ou não à aplicação de plasma. No Capítulo 1, dois tempos de aplicação foram avaliados quanto à influência do plasma na constituição físico-química da

superfície da dentina hígida e desmineralizada, na ultra morfologia da interface dente-restauração, na atividade enzimática de metaloproteinases e na resistência de união e longevidade das restaurações após um ano. Os métodos de envelhecimento *in vitro* por “exposição direta à água” e “pressão pulpar simulada” também foram comparados. O Capítulo 2 avaliou a influência da aplicação de plasma na hidrofilia da dentina, na nanodureza e módulo de elasticidade das estruturas constituintes da interface de união e na efetividade de união após dois anos de armazenamento.

2 ARTIGOS

2.1 Effect of non-thermal atmospheric plasma on bond strength, morphology and composition of dentin surface in combination with a multi-mode adhesive system.

Artigo submetido ao periódico *Clinical Oral Investigations* (Anexo I)

Autoria: Ana Paula Ayres; Jean Jacques Bonvent; Borys Mogilevych; Luis Eduardo Silva Soares; Airton Abrahão Martin; Gláucia Maria Ambrosano; Fabio Dupart Nascimento; Bart Van Meerbeek; Marcelo Giannini.

Abstract

Objectives: The aim of this study was to evaluate the effects of non-thermal atmospheric plasma (NTAP) application on dentin surface and on microtensile bond strength (μTBS) of a multi-mode adhesive system.

Materials and Methods: Human third molars had the flat dentin surface treated or not (control) with plasma for 10 or 30 s. The chemical composition of the plasma-treated dentin was determined by Raman Confocal Spectroscopy (n = 5), while the surface morphology was evaluated using the Atomic Force Microscopy (n = 3). μTBS test (n = 8) evaluated the adhesion of a multi-mode adhesive system to dentin, in etch-and-rinse (ER) and self-etch (SE) mode, after storage of specimens for 24-hour or 1-year. Bonded specimens were divided into two different aging methods: direct water exposure (DWE) and simulated pulpal pressure (SPP) and prepared for interfacial SEM investigation (n = 8). The influence of plasma on the enzymatic activity of metalloproteinases was evaluated by *in situ* zymography (n = 3). Data were analyzed using ANOVA and Tukey's tests ($p \leq 0.05$)

Results: NTAP treatment did not produce physical alteration at dentin surface. Only the amount of phosphate had a decrease in intact and etched dentin after 10 s plasma exposure. In DWE, 1-year plasma-treated specimens did not yield difference compared to control groups, whereas SPP aging strategy showed higher μTBS means for plasma-treated dentin. Enzymatic activity was treatment dependent, but control groups showed more intense fluorescence within the hybrid layer.

Conclusions: No remarkable chemical and morphological alterations were observed in dentin surface after NTAP treatments. Bonded dentin maintained μTBS values after 1-year storage. However, two different storage methods produced remarkable differences among μTBS of the groups and different distribution of failure pattern modes. In SPP evaluation, 30 s plasma treated dentin showed higher μTBS means compared to correspondent untreated groups.

Clinical Relevance: This study shows a feasible method to enhance dental adhesion, using a device commercially available. Non-thermal plasma that is generated at atmospheric pressure with relatively low input power might contribute to bonding longevity of a multimode adhesive system without provoking physical-chemical damage to dentin.

Keywords: Adhesion; multi-mode adhesive; aging methods; resin-dentin interface; enzymatic activity; atmospheric plasma.

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1. Introduction

The aging mechanisms involved in the degradation of resin-bonded interfaces occurs by a combination of mechanical stress, hydrolysis of suboptimally polymerized hydrophilic resin components and collagenolytic enzymatic activity [1,2]. The most common *in vitro* aging strategies to evaluate hydrolytic degradation of resin-dentin interfaces are thermocycling and the direct exposure of match-stick or slab in distilled water [3,4]. However, the use of simulated pulpal pressure may better simulate the *in vivo* scenario [5].

Adhesive systems interact with dentin substrate using two different approaches. The etch-and-rinse technique involves a separate acid-etching step, followed by water rinsing and the demineralized dentin is kept moist before adhesive application. The 30-40% phosphoric acid etching gel dissolves completely the smear layer and demineralizes the underlying dentin, exposing collagen fibrils [6]. A rough dentin surface is created by the acid etching process, which promotes a larger contact area between the adhesive and the substrate, increasing the bonding area.

However, literature findings indicate that etch-and-rise adhesive systems do not infiltrate the entire depth of etched dentin, leaving empty spaces between the collagen fibrils [7].

Self-etch approach uses acidic adhesive monomers that are applied to intact dentin. The acidic solution modifies the smear layer and infiltrates into partially demineralized dentin. Although they might reduce the discrepancy between etched dentin and adhesive system infiltration, some poor infiltrated areas remain [8,9], harming the bonding. This fact has been evidenced in some *in vitro* studies by decrease of dentin bond strength after storage in water for long time [10,11]. Regarding etch-and-rinse systems, the effect of acid etching and acidic primers/adhesives has been related to the enzymatic degradation of collagen fibrils, which also reduces the durability of composite restoration [12–14].

The complete encapsulation of the collagen fibrils by the adhesive resin monomers is recommended to protect them against degradation [15]. A recent review of literature has reported significant improvement of the resin bonding induced by non-thermal atmospheric plasma (NTAP) treatments [16]. Plasma is defined as partially ionized gases that contain electronically excited atoms, molecules, and ionic and free-radical species. These highly reactive particles can cross-link rapidly to form various chemical functional groups on the surface of substrates [17]. Overall, NTAP has demonstrated efficacies in improving different properties for dental bonding such as increase of dentin surface wettability [18–22], improvement of resin polymerization [23,24] and deeper adhesive penetration [18,24–26]. Another possible explanation for enhancing the adhesive-dentin bonding strength is that NTAP treatment could introduce activated sites to the dentin surface, such as free radicals or peroxides, thus enhancing adhesive monomers interaction with dentin collagen [27].

Many types of plasma jet devices have been developed in recent years and some adaptations enabled to plasma be generated at atmospheric pressure without raising the temperature of the working gas. Some investigations have found that NTAP jets can be applied to delimited sites at room temperature without damaging surrounding organic tissues [28–30], enabling thus its application in biomedical fields.

This current investigation aimed to evaluate whether NTAP treatment can promote alterations on the human dentin surface and resin bonding performance. Considering the versatility of multi-mode adhesives, the microtensile bond strength of

dentin-resin interface were tested in etch-and-rinse and self-etch approaches, in combination or not with plasma treatment. The influence of the aging strategy 'simulated pulpal pressure' compared to 'direct water pressure' was also evaluated. Additionally, *in situ* zymography was used to analyze plasma interference on enzymatic activities.

Four null hypothesis were tested: (1) Surface morphology and composition of dentin substrate would not be changed after NTAP application; (2) Plasma treatment would not influence dentin bond strength neither immediately nor after long-term water storage; (3) There would be no difference between 'direct water exposure' and 'simulated pulpal pressure' in promoting hydrolytic degradation within the resin-dentin interface after water storage for one year; (4) The collagenolytic/gelatinolytic activities after exposition to NTAP for 10 or 30 s would not be different from untreated dentin, regardless of etch-and-rinse or self-etch approach.

2. Material and Methods

2.1 Dentin preparation

Noncarious human third molars were collected under protocol (#010/2015) approved by Institutional Ethics Committee in Research (Anexo 2). Teeth were stored in 0.05% thymol solution (4^{o}C) and used within 3 months after extraction. They were cleaned and their occlusal third removed using a diamond wafering blade (Buehler-Series 15HC Diamond, Buehler, Lake Bluff, IL, USA) that was attached to an automated sectioning machine (Isomet 2000; Buehler). The sections were done under running water. A flat dentin surface was exposed and standardized smear layer created with 600-grit silicon carbide (SiC) papers using a polishing machine (APL-4, Arotec, Cotia, SP, Brazil) under water irrigation.

2.2 Non-thermal Atmospheric Plasma (NTAP) device

The plasma equipment used in this study (Surface Plasma Tool Model SAP, Surface – Engineering and Plasma Solution, Campinas, SP, Brazil) consists of a hand-held unit using Argon as operating gas at a flow rate of 5.0 liters per minute. The plasma torch emerging at the exit nozzle was about 1.0 mm in diameter and was operated at room temperature (22°C). A mobile base allowed keeping distance of 10 mm between the nozzle and the dentin surface (Figure 1), and two times of plasma

exposure (10 or 30 seconds) were used in scanning mode covering all dentin surface. Control groups did not receive plasma application.

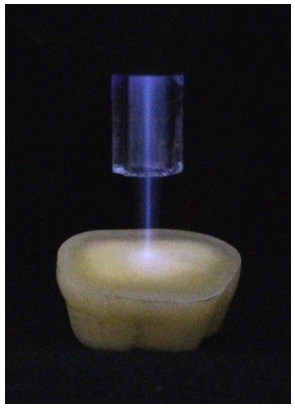

Fig. 1 Dentin surface treatment using a non-thermal argon plasma brush (Surface Plasma Tool Model SAP, Surface – Engineering and Plasma Solution LTDA, Campinas, SP, Brazil).

2.3 Atomic Force Microscopy (AFM)

The surface morphology of dentin surface was evaluated using AFM (Shimadzu/SPM 9600, Kyoto, Japan). The probe was used in the "contact" mode (n = 3), investigating surface alterations in nanoscale by repulsive electronic interactions. Its position can be recorded with the reflection of a laser on the cantilever and a photo detector, thus determining the x, y and z positions and then generating a surface profile image (20 × 20 μm). The roughness was calculated by microscope software, in agreement to Rzjis parameter (Ten Points Means Roughness). Two-way ANOVA was used to detect significant differences according to NTAP treatment or no treatment (control) in etched and unetched dentin factors ($p \leq 0.05$).

2.4 Raman Confocal Spectroscopy (RCS)

The chemical composition of the dentin treated or not with NTAP was determined by RCS (n = 5). The Raman spectra were collected using a spectrometer (Skin Analyzer – model 3510, River Diagnosis BV, Rotterdam, Netherland) with diode laser at 785 nm wavelength. The software (River Icon, River Diagnosis BV, Rotterdam, Netherland) obtained 1 spectrum of 10 seconds, with 5 μm laser penetration into the central portion of dentin surface. RCS data were analyzed by selecting spectral ranges 900-990 cm^{-1}, 990-1150 cm^{-1} and 1550-1730 cm^{-1}, corresponding to 'phosphate', 'carbonate' and 'type I collagen', respectively.

Spectrums were processed (minimum-maximum normalization, background correction, and range selection) using Opus software (OPUS v. 4.2, Bruker Optik GmbH, Ettlingen, Germany). The integrated areas of the peaks were calculated using the Microcal Origin® software (Microcal Software Inc., Northampton, MA, USA) and data were processed by two-way ANOVA Proc Mixed for repeated measures ($p \leq 0.05$). Three measurements in the same dentin slice were performed in etch-and-rinse groups: immediately after smear layer formation, after dentin acid etching and after NTAP application (10 or 30 s). The values obtained after acid etching was considered as baseline (initial) for etch-and-rinse groups while self-etch groups did not receive phosphoric acid application on dentin. Therefore, in self-etch groups the initial measurement was considered immediately after smear layer production.

2.5 Microtensile Bond Strength (μTBS) and Fracture Analysis

To evaluate the effect of NTAP on dentin bond strength, a multimode adhesive system (Scotchbond Universal, 3M ESPE, St. Paul, MN, USA) was applied in self-etch and in etch-and-rinse adhesive modes (Table 1). Ninety-six human molar teeth were randomly assigned to six groups, following the treatments: unetched dentin without NTAP application (SE control); unetched dentin + NTAP 10 s application; unetched dentin + NTAP 30 s application; etched dentin without NTAP application (ER control); etched dentin + NTAP 10 s application; etched dentin + NTAP 30 s application.

Table 1. Adhesive system, composition (batch number) and application mode.

Adhesive system / Manufacturer	Composition	Self-etch approach	Etch-and-rinse approach
Scotchbond Universal (3M ESPE, St Paul, MN, USA) pH = 2.7	1. Scotchbond Universal Etchant: 34% phosphoric acid, water, synthetic amorphous silica, polyethylene glycol, aluminum oxide (#2N0034) 2. Adhesive: 10-MDP phosphate monomer, dimethacrylate resins, HEMA, methacrylate- modified polyalkenoic acid copolymer, filler, ethanol, water, initiators, and silane (#621418)	1. Apply the adhesive to the entire preparation with a microbrush and rub it for 20 s; 2. Direct a gentle stream of air over the liquid for about 5 s until it no longer moves and the solvent is evaporated completely; 3. Light cure for 10 s.	1. Apply etchant for 15 s; 2. Rinse for 10 s; 3. Air dry 5 s; 4. Apply adhesive as for the self-etch mode.

Abbreviations: 2- hydroxyethyl methacrylate (HEMA)
10- methacryloyloxydecyl dihydrogen phosphate (10-MDP)

The dentin surface was demineralized for 15 s with 34% phosphoric acid (Scotchbond Etchant; 3M ESPE, St Paul, MN, USA) in etch-and-rinse groups. The demineralized dentin surface was blot-dried with Kimwipes tissues (Kimberly-Clark, Roswell, GA, USA). After the respective treatments, the adhesive system was applied and light cured for 10 seconds (VALO, Ultradent Products Inc., South Jordan, UT, USA). Afterwards, a composite resin block (Filtek Supreme Ultra, shade A2 enamel, 3M ESPE, St. Paul, MN, USA) was built-up (6 mm high) incrementally and each 2-mm layer was light cured for 20 seconds with the same LED light-curing unit.

Teeth from each group were randomly divided into two storage strategies: direct water exposure (DWE) and simulated pulpal pressure (SPP). In DWE, 48 bonded teeth were prepared for the microtensile test according to the no-trimming technique (n = 8). Teeth were serially sectioned vertically into 0.9 mm thick slabs with a diamond saw, under water-cooling (Isomet, Buehler; Lake Bluff, IL, USA). Each slab was then further sectioned to obtain bonded beams with a cross-sectional area of approximately 0.9 mm^2. Eight bonded beams were randomly selected per tooth and bond strength test was performed after 24 hours with four bonded beams while the other four beams were stored in deionized water for one year (DWE). Each specimen was fixed to a microtensile testing device with cyanoacrylate glue (Super Bonder, Henkel Loctite; Diadema, SP, Brazil) and tested in tension at a crosshead speed of 0.5 mm/min until failure.

The other 48 restored teeth (n = 8) were serially cut and prepared to μTBS test only after one year of water storage, accomplishing with the SPP adapted aging method, previously described by Feitosa *et al.* [31]. Briefly, the bonded teeth were covered with two coats of nail varnish; hence the passage of water was possible only through dentinal tubules. The specimens were fixed side-ways on the lid of a cylindrical receptacle, which was filled with deionized water, sealed and turned upside down to create a 20-cm water column similarly to the classic method of SPP.

After fracture, the specimens had the cross-sectional area measured at the site of the fracture with a digital caliper (Starrett; Itu, SP, Brazil) to calculate the dentin tensile bond strength. Bond strength data (MPa) was then calculated using SAS software and analyzed by split-plot two-way ANOVA (factors: treatment and storage condition) for each adhesive technique (self-etch and etch-and-rinse) and Bonferroni test ($p \leq 0.05$).

After the bond strength testing, the two ends of the fractured surfaces were mounted on brass stubs, gold-coated, and observed using scanning electron microscopy (SEM; JSM5600, JEOL Ltd, Tokyo, Japan) at magnifications of 80 ×. The failure mode of each beam was classified into categories: cohesive failure in composite resin (C); cohesive failure in dentin (D); cohesive failure in adhesive layer (A); failure between composite resin and adhesive (CA); failure between adhesive and dentin (AD); and mixed failure of composite resin, adhesive, and dentin (CAD).

2.6 Scanning Electron Microscopy (SEM)

Four bonded beams of each group obtained after tooth sectioning were used for micromorphological interfacial analysis. The beams were embedded in epoxy resin (Buehler, Lake Bluff, IL, USA) and polished with a sequence of SiC papers (600, 1200 and 2000-grits), followed by diamond pastes (6, 3 and 1 μm). Beams were rinsed and polishing debris were ultrasonically removed during 5 minutes cleaning between each polishing step. After polished, specimens were etched with 50% phosphoric acid for 15 seconds, washed, treated with 0.1% NaOCl for 10 min and re-washed. Afterwards, they were immersed in hexamethylsiloxane solution for 10 min, dried overnight (at 37^{o}C), mounted in aluminum stubs, sputter coated with gold and examined using SEM (JSM5600, JEOL Ltd, Tokyo, Japan). Representative areas of the adhesive-dentin interfaces were photographed at 500× magnification.

2.7 *In situ* zymography

Eighteen non-carious teeth were restored within ten days after extraction, following the 6 groups descriptions, and each bonded dentin/composite site was glued to a microscope slide with cyanoacrylate cement and ground down to obtain 500 μm thick specimens (n = 3). *In situ* zymography was performed with quenched fluorescein-conjugated gelatin as the MMP substrate (E-12055, Molecular Probes, Eugene, OR, USA). A 1.0 mg/mL stock solution of fluorescein-labeled gelatin, prepared by the addition of 1.0 mL water to the vials containing the lyophilized substrate were stored at -20°C until use. The gelatin stock solution was diluted 1:8 with the dilution buffer (NaCl 150 mM, $CaCl_2$ 5 mM, Tris-HCl 50 mM, pH 8.0) and an anti-fading agent was added (Mounting Medium with Dapi H-1200, Vectashield, Vector Laboratories). 50 μL of the fluorescent gelatin mixture was placed on top of

each slab and covered with a coverslip. Slides were light-protected and incubated in humidified chambers at 37°C. Briefly, hydrolysis of quenched fluorescein-conjugated gelatin substrate, indicative of endogenous gelatinolytic enzyme activity, was assessed by examination under a multi-photon confocal microscope (Zeiss, LSM 780, Carl Zeiss, Oberkochen, Germany). Optical sections of 85 µm thick were acquired from different focal planes, and the stacked images were analyzed, quantified, and processed with ZEN 2010 software (Carl Zeiss, Oberkochen, Germany).

3 Results

3.1 Atomic Force Microscopy (AFM)

Representative images of untreated and plasma-treated surfaces are shown in Figure 2. Excluding the scratches caused by 600-grit SiC sandpaper, and smear layer removal and superficial demineralization at intertubular and intratubular dentin promoted by phosphoric acid etching, no significant morphological difference can be seen among the specimens with or without plasma brush exposure. No measureable modification of average roughness (Table 2 and 3) was provoked by NTAP and results indicated no detectable damage or etching of the dentin surface.

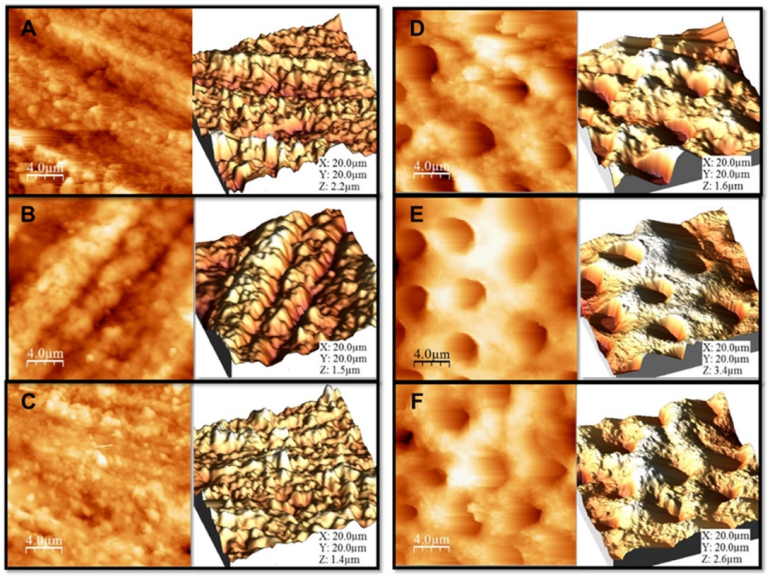

Fig. 2 3-D AFM contact mode images of dentin surface (20 × 20 µm). A: Unetched dentin pre-polished with 600-grit carbide abrasive paper without plasma application; B: Unetched dentin plasma-treated for 10 s; C: Unetched dentin plasma-treated for 30 s. D: No plasma application, dentin etched by phosphoric acid showing opened dentinal tubules; E: Etched dentin plasma-treated for 10 s; F: Etched dentin plasma-treated for 30 s.

Table 2. Root mean square (μm) and standard deviation (SD) of dentin surface.

Dentin	Control	NTAP 10 s	NTAP 30 s
Unetched	313.9 (46.3) Ab	293.6 (113.1) Ab	256.9 (91.5) Ab
Etched	361.4 (55.9) Aa	474.8 (109.5) Aa	387.4 (87.7) Aa

Means followed by distinct letters (upper case comparing treatments within the same dentin condition; lower case comparing no-etched and etched dentin within the same treatment) were statistically different ($p \leq 0.05$).

Table 3. Roughness average (μm) and standard deviation (SD) of dentin surface.

Dentin	Control	NTAP 10 s	NTAP 30 s
Unetched	257.5 (41.5) Ab	233.7 (87.4) Ab	204.2 (73.7) Ab
Etched	281.6 (45.6) Aa	385.5 (99.4) Aa	304.4 (56.8) Aa

Means followed by distinct letters (upper case comparing treatments within the same dentin condition; lower case comparing no-etched and etched dentin within the same treatment) were statistically different ($p \leq 0.05$).

3.2 Raman Confocal Spectroscopy (RCS)

Two-way ANOVA and Tukey (5%) test showed no statistical difference in carbonate and collagen type I spectrums after NTAP treatment in etched and no-etched dentin groups (Tables 4 and 5). Strong reduction of phosphate at the surface was achieved by acid etching ($p \leq 0.002$). NTAP application for 10 s affected the phosphate amount in both etched and unetched dentin surface (Table 6).

Table 4. Mean (SD) of integrated areas of Raman spectral range corresponding to carbonate in etched and unetched dentin, before and after plasma treatment (NTAP).

Dentin	Initial	NTAP 10 s	Initial	NTAP 30 s
Unetched	97.3 (13.4) a	90.6 (9.0) a	89.1 (7.8) a	93.8 (15.1) a
Etched	60.3 (6.0) b	54.8 (5.3) b	63.9 (14.2) b	64.7 (5.7) b

Means followed by distinct letters were statistically different ($p \leq 0.05$). There was no significant difference before and after plasma treatment ($p = 0.1111$ for “NTAP 10 s” and $p = 0.5760$ for “NTAP 30 s”).

Table 5. Mean (SD) of integrated areas of Raman spectral range corresponding to collagen I in etched and unetched dentin, before and after plasma treatment (NTAP).

Dentin	Initial	NTAP 10 s	Initial	NTAP 30 s
Unetched	113.9 (10.9) a	115.3 (5.5) a	116.4 (5.7) a	116.9 (2.4) a
Etched	117.9 (6.8) a	122.2 (13.4) a	118.8 (3.9) a	118.1 (19.4) a

There was no significant difference between unetched and etched dentin ($p = 0.2075$ for “NTAP 10 s” and $p = 0.7286$ for “NTAP 30 s”). There was also no significant

difference before and after plasma treatment (p = 0.5559 for “NTAP 10 s” and p = 0.9795 for “NTAP 30 s”).

Table 6. Mean (SD) of integrated areas of Raman spectral range corresponding to phosphate in etched and unetched dentin, before and after plasma treatment (NTAP).

Dentin	Initial	NTAP 10 s	Initial	NTAP 30 s
Unetched	498.6 (70.4) Aa	445.0 (32.3) Ba	456.8 (30.4) Aa	474.9 (67.1) Aa
Etched	292.6 (33.4) Ab	261.7 (23.7) Bb	315.7 (68.9) Ab	329.5 (8.1) Ab

Means followed by distinct letters (upper case comparing treatments within the same dentin condition; lower case comparing unetched and etched dentin within the same treatment) were statistically different ($p \leq 0.05$). NTAP 10 s produced statistically lower means (p = 0.0472), while no difference was found for “NTAP 30 s” (p = 0.5121).

3.3 Microtensile Bond Strength and Fracture Analysis

The mean (SD) of the μTBS outcomes are shown in Table 7. Two-way ANOVA indicated no significant difference in the μTBS after 24 hours between the adhesive approaches (p = 0.1553) and the treatments (p = 0.3931). However, after one year the two aging strategies employed in this study induced differences in terms of bonding effectiveness. The self-etch and etch-and-rinse groups treated with plasma for 30 s were characterized by a significant μTBS reduction after 1 year of DWE challenge compared to 24 hours. However, ‘NTAP 30 s’ did not differ statistically from the results of respective control and ‘NTAP 10 s’ groups at 24-hours and 1 year evaluations. Conversely, after SPP challenge, the specimens of control groups showed significant lower μTBS when compared to plasma-treated groups (10 and 30 s) in both adhesive approaches.

Table 7. Mean (standard deviation) of microtensile bond strength (MPa) as function of dentin treatment and aging strategy for each adhesive technique (self-etch = SE; etch-and-rinse = ER).

	Aging strategy		
Treatment	**24 hours**	**1 year (DWE)**	**1 year (SPP)**
SE Control	69.6(7.2) Aa	61.2(11.4) Aa	67.9(7.1) Ab
SE NTAP 10 s	73.1(3.4) Aa	70.3(12.0) Aa	73.8(8.9) Aa
SE NTAP 30 s	78.1(6.5) Aa	66.4(4.0) Ba	75.9(9.2) Aa

ER Control	71.2(5.2) Aa	73.6(8.4) Aa	65.3(5.0) Ab
ER NTAP 10 s	71.9(3.4) Aa	67.9(7.9) Aa	76.3(6.0) Aa
ER NTAP 30 s	76.7(6.2) Aa	69.4(6.8) Ba	79.8(7.8) Aa

Means followed by distinct letters (upper case comparing aging strategy within the same treatment; lower case comparing treatments within the same aging strategy) were statistically different ($p \leq 0.05$) for the same adhesive technique (SE or ER). Abbreviations: “NTAP” = Non-thermal atmospheric plasma; “DWE” = aging by direct water exposure; “SPP” = aging by simulated pulpal pressure.

The differences between SPP and DWE were also noticeable regarding the distribution of failure modes (Figure 3). In DWE, cohesive failure in composite resin was the most predominant failure pattern observed in all groups, in 24 h and 1-year evaluation. Meanwhile, for 1-year SPP groups the predominant failure pattern was adhesive (A, CA and AD).

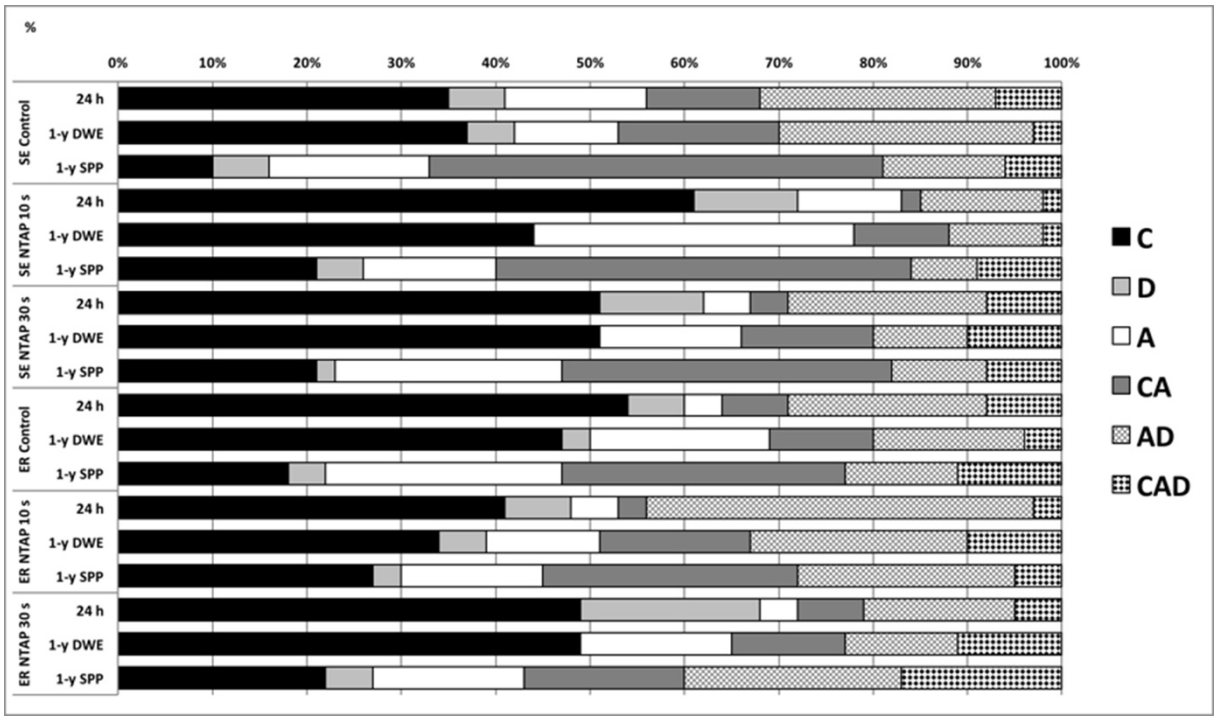

Fig. 3 Distribution (%) of failure modes of the groups tested after 24 hours (24 h) and 1-year storage in direct water exposure (1-y DWE) and simulated pulpal pressure (1-y SPP) aging mode. (C: cohesive failure in composite resin; D: cohesive failure in dentin; A: cohesive failure in adhesive layer; CA: failure between composite resin and adhesive; AD: failure between adhesive and dentin; and CAD: mixed failure of composite resin, adhesive and dentin)

3.4 Scanning Electron Microscopy (SEM)

Representative images of SEM micrographs are illustrated in Figure 4. In self-etch approach, the adhesive layer thickness is similar for the three different

groups. However, the specimens with plasma treatment showed slightly more numerous short resin tags. More contrast between the control and plasma groups was observed in etch-and-rinse specimens, once the untreated group showed a thicker adhesive layer than the counterparts with plasma treatment. In addition, the plasma-treated experimental groups clearly display longer and more numerous resin tags than the control group, even more evident after 30 s of plasma application.

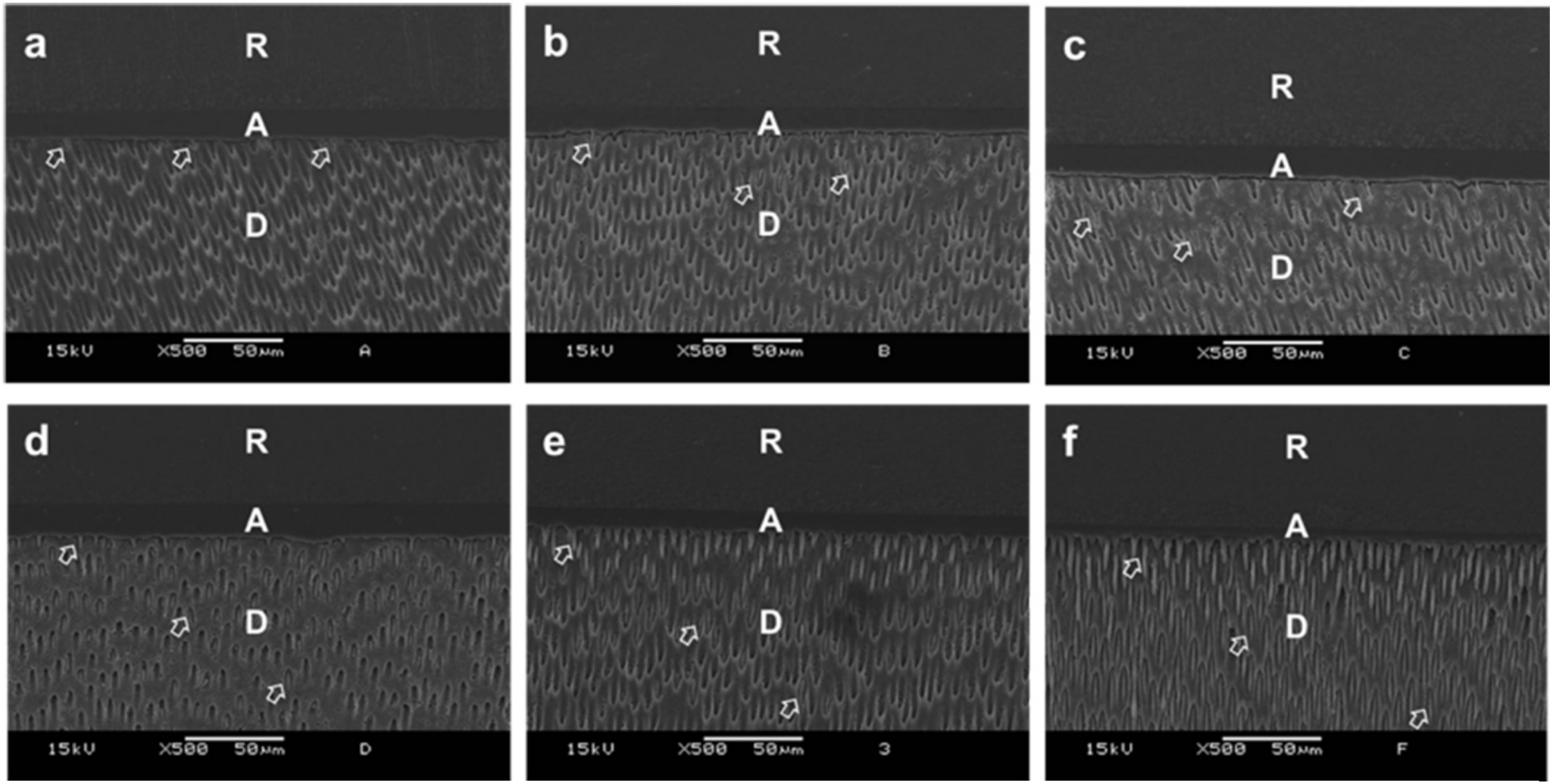

Fig. 4 Representative SEM micrographs of the adhesive/dentin interface, in self-etch (SE) and etch-and-rinse (ER) modes. Arrows indicate resin tags. (a) SE control group; (b) SE with 10 s plasma treatment; (c) SE with 30 s plasma treatment; (d) ER control group; (e) ER with 10 s plasma treatment; (f) ER with 30 s plasma treatment. (A = adhesive layer; D = dentin; R = composite resin).

3.5 *In situ* zimography

The *in situ* zimography assay showed that MMP activities significantly increased after NTAP application for 10 s in self-etch mode, when compared with the other SE groups (Figures 5A to 5C). For etch-and-rinse specimens, the *in situ* zymography revealed an intense green fluorescence in dentin, indicating that the fluorescein conjugated gelatin was strongly hydrolyzed at these sites (Figures 5D to 5F). Etch-and-rinse control group (Figure 5D) exhibited strong enzymatic activity within the hybrid layer.

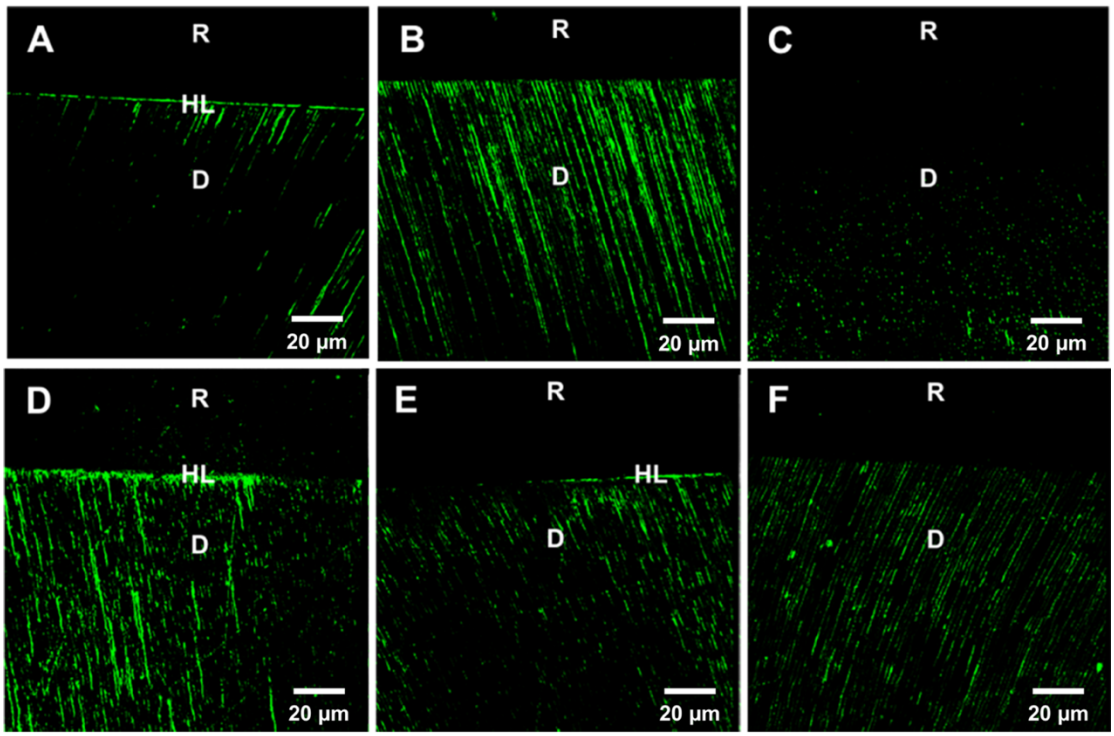

Fig. 5 Resin-bonded interface prepared with Scotchbond Universal (3M ESPE) in self-etch (A-C) and etch-and-rinse (D-F) mode and incubated with quenched fluorescein-labeled gelatin (D = dentin; HL = hybrid layer; R = composite resin). Acquired images in green channel, shows fluorescence in dentinal tubules and within the HL in self-etch control group (A); high fluorescence intensity can be observed within the underlying mineralized dentin after NTAP 10 s treatment (B), but not evidenced within the HL; lower background fluorescence was detectable in the NTAP 30 s specimen (C). Acquired images in green channel, shows fluorescence clearly detectable along the tubular wall dentin extending from the dentinal tubules, specially within the HL in etch-and-rinse control group (D); fluorescence intensity apparently gets lower in etch-and-rinse groups treated with NTAP for 10 s (E) and 30 s (F)

4. Discussion

AFM was carried out to analyze morphological changes of the dentin surface with and without plasma application and the results indicated that the

treatment did not induce physical alteration under our experimental conditions. This observation is consistent with the findings of Chen *et al.* [19] using SEM images of untreated and 30 s plasma-treated dentin surface. Sun *et al.* [32] evaluated the surface morphology and microhardness of enamel tissue, which were not influenced by plasma treatment as well. Lehman *et al.* [20] reported slight increase of surface roughness in unetched dentin, but this effect was not statistically significant in etched dentin and enamel substrates. Possible explanations for the conflicting results are the use of bovine hard tissues, differences on the setup of NTAP source operated and the use of He with H_2O_2 as feeding gas. They also did not report plasma application time to allow a direct comparison with the present study.

Regarding the chemical composition evaluation of plasma-treated dentin surface, only the amount of the element 'phosphate' decreased in intact and etched dentin after 10 s plasma exposure. The spectra of 'type I collagen' and 'carbonate' did not show alteration when compared to respective control groups. Type I collagen fibrils represents the backbone of the organic fibrillar network [33]. Chen *et al.* [27] using TEM also showed that plasma treatment did not alter the band of collagen structure. Although the same tooth was evaluated before and after plasma treatment, high standard deviation was found to 'phosphate' spectra means. This fact should infer that RCS might not be precise enough for 'phosphate' spectra evaluation on dentin surface. In another study [25], Fourier transformed infrared spectrophotometry showed increased numbers of carbonyl groups in plasma-treated demineralized dentin. In contrast, the atomic percentage of carbon (at % C) decreased in plasma-modified dentin specimens from 24,7% to 14,57% in x-ray photoemission spectroscopy (XPS) measurement [19]. It is worth mentioning that the differences in NTAP devices, settings and evaluation tests might be responsible keys to contrasting plasma effects on dental mineralized tissues. The first null hypothesis that the surface morphology and chemistry would not be changed after plasma treatment was partially rejected as alterations where found just for 'phosphate' spectra and it was plasma application-time dependent.

Ritts *et al.* [25] were the first research group to evaluate the bonding performance of a commercial adhesive with different NTAP treatment times (30 to 300 s). The bond strength of the composite restoration to peripheral dentin was significantly increased (by 64%) after 30 s of plasma treatment, while prolonged time (e.g. 5 min) resulted in a decrease in the dentin bond strength of Adper Single Bond

Plus adhesive. In the present study, no statistical μTBS difference of NTAP specimens was found in the 24 h evaluation test. Marchesi *et al.* [34] supported the use of Scotchbond Universal in self-etch approach due to improved stability after one year of water storage, when compared with μTBS results of etch-and-rinse group. However, our findings for this multimode adhesive after ageing did not show statistical differences from initial μTBS means, in neither adhesive approaches. After one year of water storage, conflicting results between the evaluated groups were found depending of the aging strategy applied.

In DWE storage, an apparent μTBS decrease was found in 30 s plasma-treated groups, but these means did not differ from the performance of the other compared groups. This negative result should be carefully considered as might be the result of two factors: high initial μTBS results for 30 s plasma-treated groups and high incidence of cohesive failures in composite resin observed in the one-year DWE aged specimens. Despite no significant statistical results were found between the groups in immediate μTBS evaluation, plasma-treated dentin for 30 s presented percentage difference in self-etch (10% higher) and etch-and-rinse (8% higher) when compared with their respective control counterparts. Meanwhile, in one-year SPP evaluation, the plasma-treated groups showed statistically higher μTBS then correspondent untreated groups, even with only 10 s of plasma application. Under this aging method, plasma treatment seems to enhance the μTBS. Therefore, the second hypothesis that plasma treatment would not influence dentin bond strength was rejected, as after one year under different aging strategies NTAP 30 s treatment affected the resin-dentin bond durability.

Other remarkable difference is that adhesive failures were observed more frequently in the specimens obtained after SPP than DWE storage method (Figure 3). High incidence of cohesive failures results in some warrant criticism in literature [35,36]. In SPP, the inner structure of the resin composite build-up is protected from direct water contact, so the water challenges directly the resin-dentin interface once it comes from exposed pulpal chamber under water column pressure. Therefore, SPP appeared more valid aging method than DWE, since the former better tested the interface properties rather than the mechanical cohesive strength of composite resin and dentin. A more recent method, mini-interfacial fracture toughness [37], enables better differentiation in bonding effectiveness between different adhesive approaches and should be used to avoid the high cohesive failure pattern found also in

immediate μTBS results, fact that make the results less reliable to be compared with aging strategies. Meanwhile, the third null hypothesis that no difference is promoted within aged resin-dentin interface between SPP and DWE after one year must be rejected.

In the literature, most of resin-dentin bonded interface results exhibits higher μTBS on NTAP-treated dentin and the majority used 30 s time application [16]. Hirata *et al.* [22] related higher μTBS for Scotchbond Universal in plasma-treated dentin in 24-h evaluation. After one year of DWE storage, no more difference was found among plasma-treated and untreated groups. Apparently, water aging negatively affected plasma-treated dentin bond but the cohesive failure pattern also increased after ageing, corroborating with our results for the same aging strategy (DWE).

Previous studies have reported that the use of NTAP prior to etch-and-rinse adhesives application increased the dentin bond strength [18,24-26,38,39], supporting the findings of the present investigation for SPP aging method. Hirata *et al.* [39] showed that NTAP dentin application after acid etching resulted in higher μTBS for XP Bond Universal adhesive system at one-week evaluation. However, this resin-dentin bond was not stable, as the μTBS decreased after one year of DWE, not differing anymore from the other groups (control and plasma pre-etching). However, the resin-dentin interface SEM micrographs of one-year stored specimens did not show detectable signs of hydrolytic degradation for any analyzed group. In the μTBS evaluation of Optibond FL, NTAP treatment and evaluation time did not influence the dentin bond strength. It was concluded that NTAP treatment influence in dentin adhesion depends on the adhesive system used and whether plasma is applied before or after dentin acid etching.

The fourth null hypothesis regarding the organic content of dentin that NTAP treatment would not promote alterations in enzymatic degradation was rejected. Mainly because the significant increase in MMP activities indicated more enzymatic activation related to plasma treatment for 10 s in mineralized dentin, although poor activity was evidenced in the hybrid layer site of this group. Previous studies have found evidence of collagenolytic and gelatinolytic activities in partially demineralized dentin treated with either etch-and-rinse or self-etch adhesives, confirming the potential involvement of these endoproteases in the disruption of incompletely resin-infiltrated collagen fibrils within hybrid layer [40,41].

Significantly higher metalloproteinases activities were detected in this assay with acid etching compared with unetched dentin groups. This finding corroborates with Mazzoni *et al.* [42] and was treatment dependent. The images suggest increased activity in areas correspondent to hybrid layer in etch-and-rinse and self-etch control groups (Figures 5A and 5D), with intense green fluorescence. It is already well established in the literature that plasma treatment increases dentinal surface energy [18–22], thus enabling adhesive monomers to promote better dissolution of smear layer and enhancing chemical properties modification of the dentin surfaces [43]. The specific device used in the present study (Surface Plasma Tool Model SAP) also showed potential in improving wettability when applied to zirconia surface [44].

It is expected that the adhesive demineralizes more an exposed dentin, provoking then more enzymatic activity than in the case of not totally dissolved smear layer, which would still partially cover the superficial dentin. In this way, the organic content of covered dentin would be less affected by functional monomer chemical interactions. This theory is supported by less collagenolytic activity evidenced in the area correspondent to hybrid layer when compared to the self-etch control group (Figure 5A). Mine *et al.* [45] studied the effect of Clearfil S3 Bond on different types of dentin surface-preparation methods. It was reported that the adhesive did not dissolve the bur cut, nor the SiC-ground smear layer, but impregnated it. In the present study, we also used a 10-MDP-based 'ultra-mild' (pH ≈ 2.7) one-step adhesive and the dentin surface was prepared with 600-grit SiC paper thus producing a 'thick' smear layer. It can be speculated that SBU adhesive might not completely infiltrate the SiC-ground dentin surface on SE control group, producing more enzymatic activity in this hybrid layer area.

A possible explanation for the enzymatic stimulation after 10 s of plasma application in intact dentin (Figure 5B) is that this short application time might induce photobiostimulation or photobiomodulation of metalloproteinases. Low energy doses application can alter enzymatic processes [46] as in low intensity laser (LIL) therapy. The energy absorbed by the living tissues increases the kinetic energy at molecular and cellular level, and the quantity of delivered energy determines the final effect [47]. There is an optimal dose of light for each particular application and doses higher or lower than this optimal value may have no therapeutic effect. A less than optimal choice of parameters can result in reduced effectiveness of the treatment, or even a

negative therapeutic outcome [47]. This concept has been used as explanation for conflicting results in LIL and might also be valid to NTAP, as this technology also delivers energy and light, producing different wavelengths. Nevertheless, after one year of SPP storage, 10 s plasma treatment still exhibited higher values of μTBS than SE and ER control groups, not differing from 30 s plasma treatment results, which could implicate in no significant collagen fibrils degradation over time.

As plasma treatment for 10 s apparently produced more enzymatic activity than the corresponding untreated control dentin, it would be logically expected that a prolonged time of plasma application would generate even more intense green fluorescence. On the contrary, zymography images showed the lowest collagenolytic/gelatinolytic activity for this experimental group in self-etch adhesive technique (Figure 5C). One could speculate that this contradictory result may be explained by the fact that a longer plasma treatment time (30 s) could provoke the opposite effect, leading to cell activities inhibition. In fact, studies have showed that plasma treatments reduced the expression and activities of MMP-2 and MMP-9 on thyroid papillary cancer cell [48] and on multiple myeloma cells [49]. The down-regulation of metalloproteinases by NTAP would be a promising tool to avoid enzymatic degradation of dentin over time.

The etch-and-rinse adhesive technique revealed stronger hydrolyzing of fluorescein conjugated gelatin than self-etch zymography images. These results agree to Mazzoni *et al.* [42] findings and that difference may be due to the fact that pre-etched dentin treated with etch-and-rinse adhesives simply exposes more dentin matrix than occurs using self-etching approach. Differences were also found between experimental and control groups. Plasma application apparently inhibited the enzymatic activity in etch-and-rinse specimens, specially in the hybrid layer area (Figures 5E and 5F). A decreasing gradient of resin monomer diffusion within the acid-etched dentin results in incompletely infiltrated zones along the bottom of the HL that contain denuded collagen fibrils [50–53]. The high fluorescence in the area correspondent of HL found in control group might represent sites of collagen fibrils not encapsulated by monomers. These unprotected collagen fibrils presumably become more susceptible to enzyme degradation, phenomenon previously described by other authors specially for simplified adhesives [54–57]. In contrast, almost no fluorescence was detected in the same region for plasma-treated demineralized

groups, corroborating our hypothesis that plasma treated-dentin should present a better resin monomer infiltration and polymerization.

SEM interfacial analysis clearly showed more intensive adhesive penetration after plasma application on demineralized dentin, showing more numerous and longer resin tags, specially for 30 s treatment time (Figure 4F). Previous reports are in agreement with these findings [18,24–26]. However, whether more dentin matrix exposition by etch-and-rinse adhesives induces more enzymatic activity, it would be expected that the groups presenting more dentin tubules fulfilled by thicker resin tags would also present more collagenolytic activity, once the acidic monomers would be in more intimate contact with a larger area of collagen fibrils meshwork. Unlikely, such correlation was not observed. Future researches might be encouraged to discover if the plasma modification on demineralized dentin creates well-developed resin tags that seal the hydrophilic domains within the adhesive complex and whether promotes inactivation or silencing of collagenolytic enzymes, improving thus the durability of resin-dentin bonds.

The multi-mode adhesive evaluated in this study, Scotchbond Universal contains 10-MDP in its composition (Table 1). The ionic bond formation of 10-MDP with Calcium ions from hydroxyapatite remaining at the interface is considered one of the most important factor for the benefit of bonding durability [58,59]. In addition, the presence of dense self-assembled nano-layers exhibiting hydrophobic nature might help against hydrolytic degradation of adhesive interface. It is also expected that the collagen is better protected as the hybrid layer formed by 10-MDP-based adhesive keep the hydroxyapatite crystals. This functional monomer hardly dissolves apatite crystals and forms more stable calcium phosphate salt [60]. Since there was no significant difference in dentin bond strength after one-year water storage, in DWE and SPP modes, it can be assumed that 10-MDP might have contributed to this long-term durability for all evaluated groups. Reactive ions and radicals developed in dental surfaces by plasma could have introduced them new functionalities, such as new chemical structures to interact with adhesive components. This would also be favorable to release more Calcium ions from hydroxyapatites to interact with functional monomers, and then producing more consistent self-assembled nano-layers.

Yoshida *et al.* [61] using XRD revealed Ca-salt formation and nano-layering at dentin with the one-step adhesive Scotchbond Universal and these

findings were fully corroborated by scanning transmission electron microscopy (STEM). If nano-layering does improve bond durability, one should explore whether plasma activation could intensify this chemical formation. For instance, the areas where it was observed more intense nano-layering were in more etched/demineralized dentin, probably because more Calcium ions have been extracted, and also in the neighborhood of dentinal tubules, where the pre-existing and/or remained water may have ionized more the functional monomer [61]. It can be speculated that the better bonding performance of plasma-treated groups after one year of storage in SPP method, compared to respective control groups, might be associated to plasma activation of dentin surface. As consequence, more Calcium ions are released to chemically bond with 10-MDP, then producing more intense nano-layering and contributing to the bond durability. However, the complex molecular interactions at the plasma-treated dentin interface have hardly been investigated yet and are far from being understood.

Regarding the chronic hydrolysis of resin components caused by water sorption and/or esterases [2], as NTAP increases the hydrophilicity of the dentin surface, the accumulation of hydrophilic monomers and water may impair the polymerization reaction of the monomer adhesives, reducing the dentin bond strength over time. Meanwhile, evidences of polymer degradation were not reported, under the limitations of the present study and comparing only one year of water storage. Unlike other studies [18,25,38], the dentin was not rewetted after NTAP application. Kim *et al.* [26] related that plasma-drying could be a promising method to control the moisture and improve the penetration of adhesive and the mechanical property of adhesive/dentin interface.

Although some conflicting results were presented to plasma treatment conditions, this pioneer study shows a feasible and useful method to enhance dental adhesion, using a device commercially available. Further studies may be developed to prove that non-thermal plasmas that are generated at atmospheric pressure with relatively low input power would be suitable for clinical practice, in combination with different adhesive systems.

5. Conclusion

The following conclusions are addressed from the results of the current study:

1) AFM images indicated that no significant morphology change was induced by plasma treatment;

2) No remarkable chemical changes were observed in dentin after plasma 30 s application. However, plasma treatment for 10 s decreased the percent of phosphate element in etched and no-etched dentin surface;

3) SEM dentin-resin interfacial analysis showed more numerous and longer resin tags in plasma-treated acid-etched dentin and a thinner adhesive layer, specially for NTAP 30 s;

4) After storage for one year, Scotchbond Universal maintained bond strength values, with no differences between self-etch and etch-and-rinse approaches. However, 'Direct Water Exposure' and 'Simulated Pulpal Pressure' storage methods produced remarkable statistical differences among μTBS of the groups and different distribution of failure modes;

5) *In situ* zimography showed that MMP activities might be affected by NTAP application and the results depends on plasma application time and adhesive technique employed.

Compliance with Ethical Standards

Conflict of Interest: Author Ayres A.P. declares that she has no conflict of interest. Author Bonvent J.J. declares that he has no conflict of interest. Mogilevych B. declares that he has no conflict of interest. Soares L.E. declares that he has no conflict of interest. Author Marin AA declares that he has no conflict of interest. Author Ambrosano G.M. declares that she has no conflict of interest. Author Nascimento F.D. declares that he has no conflict of interest. Author Van Meerbeek B. declares that he has no conflict of interest. Author Giannini M. declares that he has no conflict of interest.

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Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: For this type of study, formal consent is not required.

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2.2 Benefits of non-thermal atmospheric plasma treatment on dentin adhesion

Artigo a ser submetido ao periódico *Operative Dentistry*

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Abstract

Purpose: This study aimed to evaluate the influence of two non-thermal atmospheric plasma (NTAP) application times and two storage times on microtensile bond strength (μTBS) to dentin. The influence of NTAP on mechanical properties of dentin-resin interface was studied by analyzing nanohardness and Young's modulus. Water contact-angles of pre-treated dentin and hydroxyapatite were also measured to assess possible alterations in surface hydrophilicity upon NTAP.

Methods: Forty-eight human molars (n = 8) were used in μTBS after partial crown removal to expose flat dentin surfaces. One half of each dentin surface received phosphoric acid conditioning, while the other half was not etched and covered with metallic paper during the acid application. Afterwards, NTAP was applied on the entire dentin surface (etched or not) for 10 or 30 s. Control groups did not receive NTAP treatment. The multimode adhesive Scotchbond Universal (SBU, 3M ESPE) was applied and light-cured using a polywave LED curing light VALO (1,200 mW/cm^2, Ultradent Products Inc.). A composite (Filtek Supreme Ultra, 3M ESPE) build-up was made in layers. After 24 h of water storage at 37^oC, the specimens were sectioned perpendicular to the interface to obtain approximately six specimens or bonded beams (approximately 0.9 mm^2 at cross-sectional area) representing the etch-and-rinse (ER) approach and another six specimens from self-etch (SE) approach, treated or not with NTAP. Specimens were tested in tension immediately after sectioning or after storage for two years in deionized water. Other three bonded teeth were selected for each group for nanoindentation methodology to obtain the nanohardness and Young's modulus values (n = 3). Water contact-angle analysis was conducted using CAM200 goniometer. Droplet images of dentin and hydroxyapatite surfaces with or without 10 or 30 s plasma treatment were captured at different water-deposition times (5 to 55 s).

Results: Two-way ANOVA revealed significant differences in μTBS after 2 years of water storage in SE approach. Plasma-treated groups means were not statistically different from respective initial results in both approaches. Meanwhile, after two years

of aging, SE control group showed a decrease from initial μTBS and presented a mean statistically lower than 'NTAP 30 s' group in ER approach. Nanohardness and Young's modulus for the hybrid layer were significantly higher for NTAP-treated groups. The influence of NTAP treatment in hydrophilicity was more evident in hydroxyapatite samples. Dentin hydrophilicity slightly decreased after 10 s of NTAP, but the difference was higher when the plasma was used for 30 s.

Conclusions: Dentin NTAP treatment contributed to the bonding-degradation retarding effect. This longevity might be correlated to the increase of nanohardness and Young' modulus of the hybrid layer and to the better adhesive infiltration, once dentin hydrophilicity was also improved. Although some effects were observed using NTAP for 10 s, the results suggest that 30 s is the most indicated treatment time.

Clinical Relevance: The application of non-thermal atmospheric plasma onto dentin for 30 s improved the longevity of a multimode adhesive after two years of water aging. This result might be related to the higher nanohardness and Young's modulus of hybrid layer and greater hydrophilicity of plasma-treated dentin.

1. Introduction

One of the most recent innovations in Adhesive Dentistry involves the treatment of different dental surfaces using non-thermal atmospheric plasma (NTAP), a novel technology that delivers highly reactive species in a gaseous medium at or below physiologic temperature. This technology try to solve challenges commonly associated with hybridization of dentin during bonding procedures, therefore influencing the quality and longevity of the tooth-resin interface. A recent published review collected and summarized the current advances of NTAP in improving the durability of dentin bonding (1). The studies have demonstrated that NTAP applied on etched dentin surface enhanced the bond strength of etch-and-rinse adhesives (2–4), but the results were more product dependent when employed to self-etch adhesives (5,6).

Overall, NTAP has demonstrated efficacies in improving different properties for dental bonding because provides higher wettability of dentin surface (2,6–9), improves the resin polymerization (10,11) and allows deeper adhesive penetration (2,4,11). For etch-and-rinse bonding technique, it was reported that a short plasma treatment could change the chemical structure of the exposed collagen

fibrils and increase the dentin surface hydrophilicity, which was related to allow better adhesive penetration into dental collagen fibrils and enhanced the dentin bond strength (11).

A complex biomechanical entity is formed in adhesive dental restoration that consists of tooth substrate and the biomaterial. In order to predict the long-term performance of dental adhesives, it is necessary to understand their mechanical properties. The bonding area between the restorative resin composite and the dentinal cavity wall presents a gradual transition of different components resulting in a heterogeneous gradient of physic-mechanical properties (12). Nevertheless, acid pre-treatment modifies the hardness of dentin surface and the bonded interface zone might allow some flexibility with hybridization process after resin polymerization. Such elastic bonding area might have a strain capacity sufficient to relieve stresses between the composite shrinkage and the rigid dentin substrate (12,13), thereby preserving the integrity of marginal adaptation and consequently increasing the restoration durability.

The influence of NTAP application on the mechanical properties of the adhesive-dentin interface was not established yet. NTAP effect depends on treatment time, working gas, input power, pulse frequency of the plasma device and also on other factors related to the substrate (1). Some investigations relate wettability enhancement associated to NTAP treatment in different dental substrates (2,6–9). The plasma device of the present study (Surface Plasma Tool Model SAP; Surface – Engineering and Plasma Solution, Campinas, Brazil) improved the wettability of zirconia surface, decreasing the contact angle approximately 50% (14). But still lack information of the influence of this specific equipment and settings on the hydrophilicity of dentin tissue.

Therefore, this study aimed to assess to which extent two times of NTAP application, 10 and 30 seconds, affect the long-term bonding effectiveness in terms of microtensile bond strength (μTBS) of one commercial multimode adhesive system. The influence of NTAP on mechanical properties of dentin-resin interface was studied by analyzing nanohardness, Young's modulus and contact angle (CA). The following null hypotheses were tested: (1) two years of water storage would not decrease μTBS to plasma-treated dentin: (2) NTAP would not affect the nanohardness and Young's modulus of resin-dentin interface' structures (dentin,

hybrid layer and adhesive layer) and (3) dentin and hydroxyapatite surface hydrophilicity would not be affect by NTAP treatment.

2. Materials and Methods

2.1 Microtensile bond strength

Forty-eight noncarious human third molars (approved by the Commission for Medical Ethics of KU Leuven under protocol number S57622) were stored in 0.5% chloramine/water at 4^{o}C and used within 3 months after extraction. The occlusal third of the crowns were removed with a diamond saw, exposing the occlusal dentin surface (Isomet 100; Buehler, Lake Bluff, IL, USA). A standardized smear layer was produced under water irrigation using 320-grit SiC paper (Buehler-Met II, Buehler, Lake Bluff, IL, USA) and the flat surface was divided in two parts with similar area using a thin diamond blade. One half of each dentin surface was demineralized for 15 s with 34% phosphoric acid (Scotchbond Etchant; 3M ESPE, St Paul, MN, USA), while the other half was covered with metallic paper and was not etched. Afterwards, NTAP was applied on the entire dentin surface (etched or not) for 10 or 30 s, as described in detail in the previous study (15). Control groups did not receive NTAP treatment. Thus, the following groups were investigated (n = 8):

1- Etched dentin (control 1);

2- Unetched dentin (control 2);

3- Etched dentin + NTAP for 10 seconds;

4- Unetched dentin + NTAP for 10 seconds;

5- Etched dentin + NTAP for 30 seconds;

6- Unetched dentin + NTAP for 30 seconds.

After the respective treatments, a multimode adhesive system (Scotchbond Universal, 3M ESPE, St. Paul, MN, USA; Table 1) was applied following the manufacturers' instructions and light-cured with a polywave LED unit (VALO, Ultradent Products Inc., South Jordan, UT, USA) with an output of around 1,200 mW/cm^2, as measured by the MARC Patient Simulator (BlueLight Analytics, Halifax, NS, Canada).

A composite buildup (Filtek Supreme Ultra, shade A2 enamel, 3M ESPE, St. Paul, MN, USA) was made in layers (6 mm high) and each 2-mm layer was light cured for 20 seconds with the polywave LED light-curing unit VALO (1,200mW/cm^2;

standard mode; Ultradent Products Inc., South Jordan, UT, USA). The tooth root was removed four mm below the adhesive-dentin interface. After 24 h of deionized water storage (37^{o}C), the specimens were sectioned perpendicular to the interface into 0.9 mm thick bonded beams (specimens) with a diamond saw, under water-cooling (Isomet, Buehler; Lake Bluff, IL, USA). Approximately six specimens representing the etch-and-rinse approach and another six specimens from self-etch approach were obtained after sectioning the teeth. After one week, beams were attached to a BIOMAT jig (16) with cyanoacrylate glue (Model Repair II Blue; Dentsply-Sankin, Tochigiken, Japan) and tested in tensile at a crosshead speed of 1 mm/min until failure in a universal testing machine (LRX; Lloyd, Hamshire, UK). The specimens from the other twenty-four teeth were stored in deionized water for two years. A single failure stress value was calculated for each half of the tooth by averaging all beams obtained from that half tooth (n = 8). μTBS (MPa) was derived from dividing the impose force (Newton) at the time of fracture by the bonded area, which cross-sectional area was measured with a digital caliper (Starrett; Itu, SP, Brazil). Bond strength data (MPa) was then calculated using R software and analyzed by split-plot two-way ANOVA (factors: treatment and storage time) for each adhesive technique (self-etch and etch-and-rinse) and Tukey's multiple comparison test ($p < 0.05$).

Table 1. Composition of the adhesive system (batch number)

Adhesive system / Manufacturer	**Composition**
Scotchbond Universal pH = 2.7	1. Scotchbond Universal Etchant: 34% phosphoric acid, water, synthetic amorphous silica, polyethylene glycol, aluminum oxide (#N489165) 2. Adhesive: 10-MDP phosphate monomer, dimethacrylate resins, HEMA, methacrylate-modified polyalkenoic acid copolymer, filler, ethanol, water, initiators, and silane (#569736)

Abbreviation: 2- hydroxyethyl methacrylate (HEMA).
10- methacryloyloxydecyl dihydrogen phosphate (10-MDP)

2.2 Nanohardness and Young's modulus

The same groups were used in this part of the study (n = 3). Three bonded teeth from each group were longitudinally sectioned through the sample center with a diamond saw, under water-cooling (Isomet, Buehler; Lake Bluff, IL, USA) to obtain two 1.5-mm-thick bonded slices. Each central slab was individually embedded in an epoxy resin (EpoxiCure, Buehler, Lake Bluff, IL, USA) and manually polished under water irrigation using SiC paper (Buehler-Met II, Buehler, Lake Bluff, IL, USA) with decreasing abrasiveness (600, 1000, 1200, 1500, and 2000). Special soft discs (Apex Diamond Grinding Disks, Buehler, Lake Bluff, IL, USA) were associated with diamond polishing suspensions (MetaDi, Buehler, Lake Bluff, IL, USA) of 9, 6, 3, 1, and 0.5 μm grit size. Samples were ultra-sonically cleaned with distilled water for 5 min between each polishing step.

The computer-controlled nano-indenter Hysitron Custom Triboindenter (Hysitron, Minneapolis, MS, USA) was used with a cell Berkovich point for the nanohardness and Young's modulus evaluation. Samples were individually placed on a computer-controlled X-Y table and were kept hydrated during the test. To ensure a precise transfer of the pre-programmed positions to the nano-indenter, an accurate calibration of the probe was performed on the standard fused quartz sample before the test' start. Five equally spaced (10 μm) indentations were pre-programmed and performed for the dentin, hybrid layer and adhesive layer, totalizing fifteen per specimen, with a load of 1000 μN and a standard trapezoidal load function of 5-2-5 s. The nanohardness and Young's modulus of each area were computed as described elsewhere (17). Data were analyzed using two-way ANOVA and Tukey's test ($p < 0.05$).

2.3 Contact angle (CA) by sessile drop method

2.3.1 Human teeth

Noncarious human third molars were selected and stored in 0.5% chloramine/water (4^{o}C) to be used within 3 months after extraction. The teeth had their root and occlusal third removed using a diamond wafering blade (Buehler-Series 15HC Diamond, Buehler, Lake Bluff, IL, USA) on automated sectioning device (Isomet 2000; Buehler, Lake Bluff, IL, USA) under running water. The exposed surface was ground with 600-grit SiC paper (Buehler-Met II, Buehler, Lake Bluff, IL,

USA), under water irrigation. All dentin slices surfaces were carefully verified, by stereomicroscopy, for the absence of enamel/pulp tissue (Wild M5A, Wild, Heerbrugg, Switzerland).

Half of each tooth (n = 5) was treated with NTAP brush for 10 or 30 s whilst a blade was used to separate and protect the other half side, which was used as untreated same-tooth control. This split of the sample was considered important once the standard deviation within dentin is too big as it depends on different factors as depth, age of the teeth and number of tubules (18).

2.3.2 Hydroxyapatite plate

Commercially available hydroxyapatite plate (10 × 10 × 2 mm; APP100, Hoya, Tokyo, Japan) with total area similar to an entire dentin flat slice dimension were prepared for contact angle evaluation. The purpose was to analyze an inorganic material present in dentin with and without NTAP application. Comparing the results with dentin hydrophilicity should then estimate plasma influence in total and partial inorganic material. The blocks were also divided in two halves and a blade protected one half from plasma treatment (control) during the 10 or 30 s application.

2.3.3 Contact angle measurement – 3 repetitions in the same spot

Excess water on dentin surfaces was gently blot-dried with Kimwipes tissues (Kimberly-Clark, Roswell, GA, USA) before water CA measurement. Hydroxyapatite blocks were air-blow cleaned and kept dried before the experiment. The CA of distilled water was measured by the sessile drop technique with the use of CAM200 goniometer (KSV Nima, Espoo, Finland) and the samples were kept in a 100% humidity chamber during measurement.

A drop of water (approximately 1.0 μL) was placed on one of the halves of dentin surface (n = 3) and the image was immediately sent via the camera to the computer for analysis. Images were captured every 5 s at different water-deposition times (5 - 55 s).

Specimen images were analyzed by a computer program (Image J software) with an angular dimension tool to measure the static CA. Right and left angles were measured to obtain a mean CA value. One drop of water was applied on each half of sample surface; treated and control side. After the first CA measurement, the sample was kept in position, blot-dried and another drop of water

was applied in the same spot, following the same protocol. All measurements were done in triplicate.

2.3.4 Contact angle measurement – Immediate analysis (unrepeated)

The intriguing results of contact angle data after 3 repetitions, let us decide to perform another test, recording the CA immediately after plasma treatment, evaluating only one drop of water. In this way, it was possible to assess better the surface hydrophilicity change upon treatment, without water intake interference. Therefore, ten dentin and hydroxyapatite samples (n = 5) were prepared as described before and the CA were individually evaluated in each half of the specimens.

3. Results

3.1 Microtensile bond strength (μTBS)

No significant difference was found to the factor ‘evaluation time’ (p = 0.22) in etch-and-rinse approach, while factor ‘treatment’ (p = 0.02) and the interaction ‘evaluation time’ and ‘treatment’ (p < 0.00) showed statistical differences. In self-etch approach, the factor ‘evaluation time’ was statically significant (p < 0.00), while no difference was found to the factor ‘treatment’ (p = 0.20) and the interaction ‘evaluation time’ and ‘treatment’ (p = 0.17). At 1-week evaluation, no differences among the treatments were found in both adhesive techniques (Figure 1). After 2 years of water storage in etch-and-rinse evaluation, ‘NTAP 30 s’ group presented higher mean of μTBS compared to control group, not differing from ‘NTAP 10 s’. In self-etch evaluation, after aging the control group showed a decrease from initial μTBS mean, while ‘NTAP 30 s’ and ‘NTAP 10 s’ groups kept stable results.

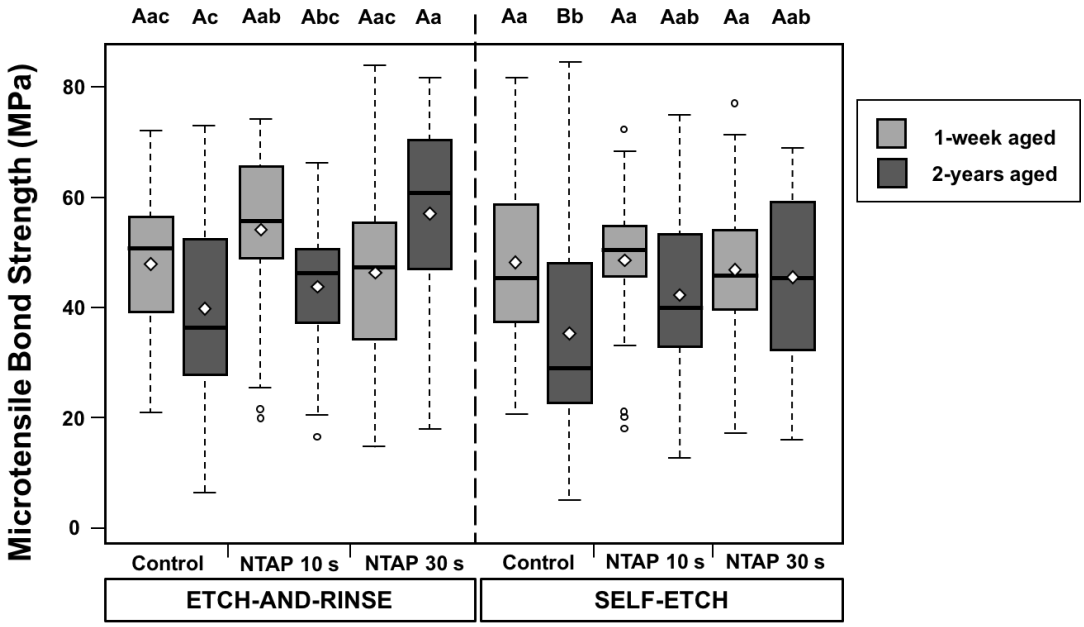

Figure 1. Boxplots represent the μTBS of untreated (control) and plasma-treated dentin (NTAP for 10 or 30 s) groups. Means followed by distinct letters (upper case comparing evaluation time; lower case comparing treatments) were statistically different (Tukey's test, $p < 0.05$) for the same adhesive technique (etch-and-rinse or self-etch).

3.2 Nanohardness and Young's modulus

Mean values and standard deviation of nanohardness and Young's modulus are shown in Table 2 and 3, respectively. The nanohardness of 'dentin' (p = 0.295) and 'adhesive layer' (p = 0.181) did not show significant differences among groups. In 'hybrid layer', control groups showed lower nanohardness than correspondent experimental groups.

The lowest dentin Young's modulus was recorded for 'NTAP 30 s' self-etch group, although not differing from NTAP 10 s self-etch and NTAP 30 s etch-and-rinse groups. For both approaches (etch-and-rinse and self-etch) the groups treated with NTAP (10 and 30 s) showed higher Young's modulus than respective controls. There was no statistical difference among groups in 'adhesive layer' evaluation (p = 0.062).

Table 2. Nanohardness (GPa) means (standard deviation) of the dentin, hybrid layer and adhesive layer (n=3) of a multi-mode adhesive. The dentin was either plasma treated (NTAP 10 or 30 s) or untreated (control) in self-etch and etch-and-rinse approaches.

Treatment	Dentin	Hybrid Layer	Adhesive Layer
Control SE	2.6 (0.5) a	1.4 (0.1) b	1.3 (0.0) a
SE NTAP 10 s	2.4 (0.1) a	1.6 (0.1) a	1.3 (0.0) a
SE NTAP 30 s	2.3 (0.2) a	1.6 (0.3) a	1.4 (0.1) a
Control ER	2.5 (0.1) a	1.2 (0.1) b	1.3 (0.0) a
ER NTAP 10 s	2.7 (0.4) a	1.5 (0.1) a	1.3 (0.0) a
ER NTAP 30 s	2.0 (0.1) a	1.4 (0.1) a	1.3 (0.1) a

Identical letters in same column did not differ by Tukey's test ($p > 0.05$) (NTAP = non-thermal atmospheric plasma; Control SE = self-etch dentin specimens without NTAP; Control ER = etch-and-rinse dentin specimens without NTAP).

Table 3. Young's modulus (GPa) mean values (standard deviation) of the dentin, hybrid layer and adhesive layer (n = 3) of a multi-mode adhesive. The dentin was either plasma treated (NTAP 10 or 30 s) or untreated (control) in self-etch and etch-and-rinse approaches.

Treatment	Dentin	Hybrid Layer	Adhesive Layer
Control SE	36.7 (4.9) a	15.6 (2.1) b	10.0 (0.6) a
NTAP 10 s	35.5 (4.5) ab	20.1 (1.5) a	10.1 (0.6) a
NTAP 30 s	33.4 (5.6) b	21.0 (4.2) a	10.1 (0.9) a
Control ER	37.3 (2.6) a	12.2 (0.5) b	10.1 (0.3) a
NTAP 10 s	37.7 (2.1) a	15.1 (1.8) a	10.4 (0.1) a
NTAP 30 s	33.4 (4.0) a	14.7 (1.2) a	10.7 (1.2) a

Identical letters in same column did not differ by Tukey's test ($p > 0.05$). (NTAP = non-thermal atmospheric plasma; Control SE = self-etch dentin specimens without NTAP; Control ER = etch-and-rinse dentin specimens without NTAP).

3.3 Contact angle by sessile drop method

3.3.1 Contact angle measurement – 3 consecutives repetitions in the same spot

The contact angles of NTAP 30 s treated specimens were significantly lower than those on untreated dentin/hydroxyapatite surfaces (Figures 2 and 3). Dentin hydrophilicity slightly decreased after 10 s of plasma treatment time, but the difference from dentin control group was higher when the plasma was used for 30 s (Figure 2). The influence of NTAP treatment in hydrophilicity was more evident in

hydroxyapatite samples (Figure 3), in which the contact angle deeply decreased even with just 10 seconds of NTAP application. In CA measurement repetitions, dentin/hydroxyapatite control groups remained close to the first CA means while 'NTAP 30 s' groups showed gradually higher CA results, both in dentin and hydroxyapatite.

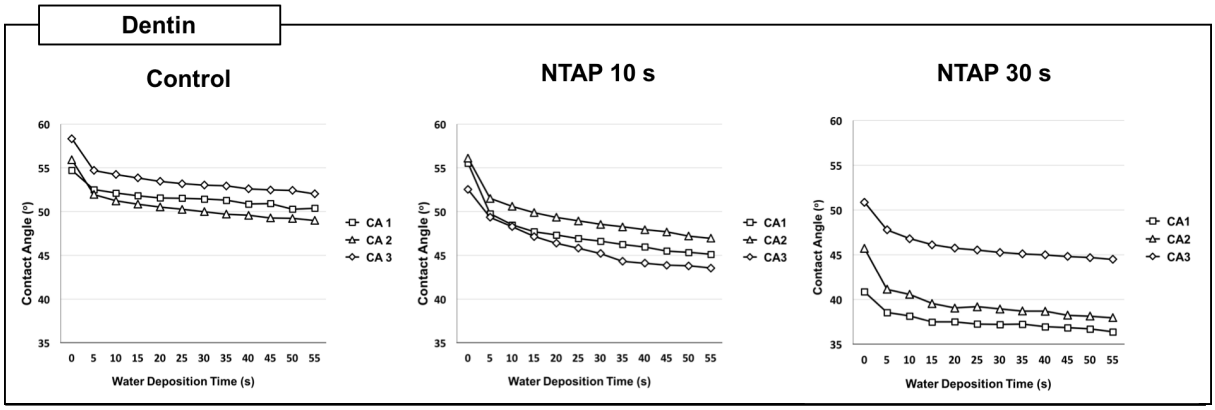

Figure 2. Means of contact angle of water on dentin treated with either NTAP for 10 or 30 seconds, as compared with that on untreated dentin (Control). (CA1; CA2; CA3 = first; second and third contact angle measurement, respectively).

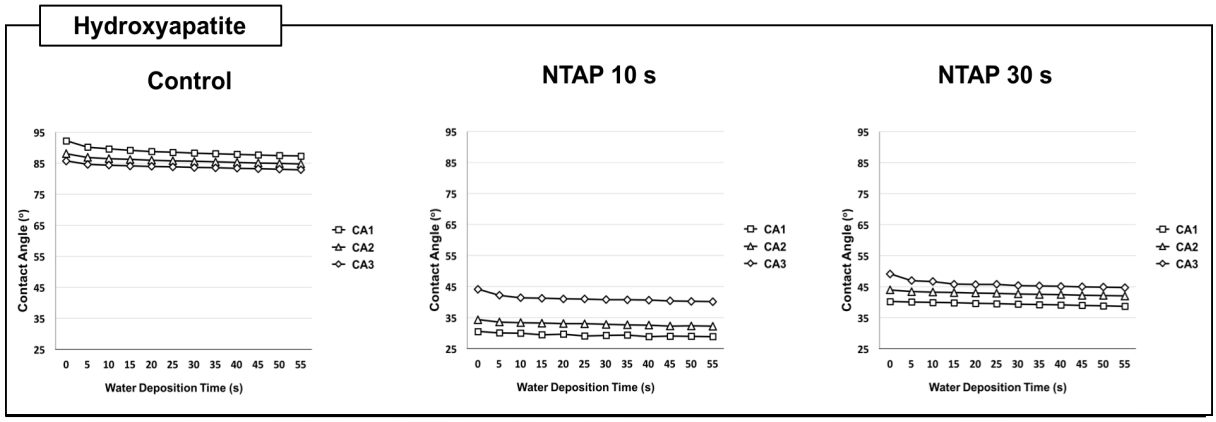

Figure 3. Means of contact angle of water on hydroxyapatite treated with either NTAP for 10 or 30 seconds, as compared with that on untreated hydroxyapatite (Control). (CA1; CA2; CA3 = first; second and third contact angle measurement, respectively).

3.3.2 Contact angle measurement – Immediate analysis (unrepeated)

The Figure 4 shows dentin' and hydroxyapatite' CA means of immediate plasma-treated and untreated (control) surfaces. In dentin, the discrepancy between 'NTAP 30 s' and control group was greater than the difference between 'NTAP 10 s' and respective control group. However, in hydroxyapatite substrate both treatment' times produced large difference in CA compared to control group means.

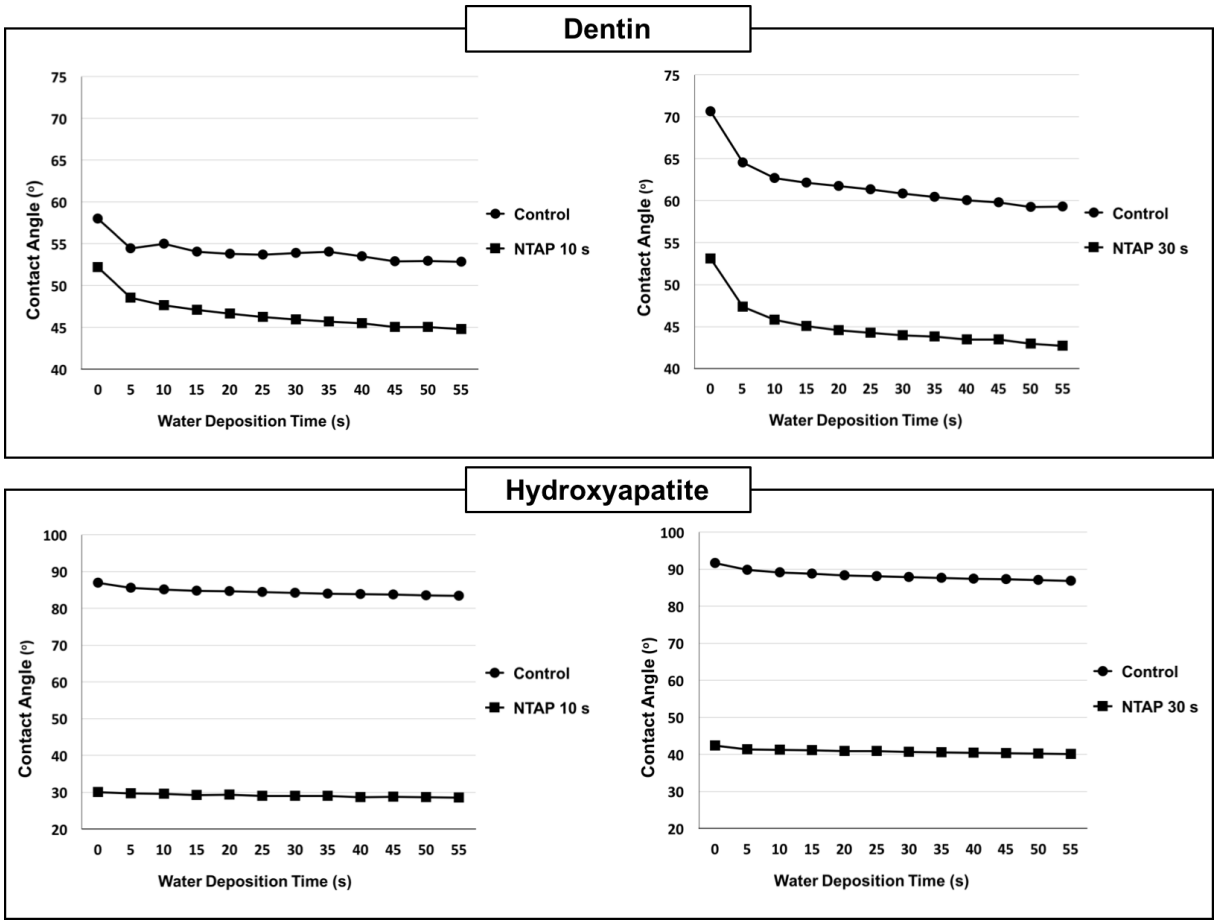

Figure 4. Means of progression in contact angle immediately after NTAP application for 10 or 30 seconds, as compared with that on untreated (Control) dentin and hydroxyapatite.

4 Discussion

The benefits of applying plasma to dentin tissue are expected to act primarily on the longevity of adhesive restoration. Therefore, it is necessary to evaluate such effects in function of the aging of the tooth-restoration interface. The first null hypothesis was accepted because the μTBS of plasma-treated dentin did not decrease after two years, neither in self-etch nor etch-and-rinse approach.

Regarding the multimode adhesive evaluated in the present study, two years of water aging did not produce significant μTBS values reduction statistically in ER approach, however the factor ‘evaluation time’ was significant in SE evaluation. Scotchbond Universal (SBU) adhesive long-term effectiveness presents some conflicting results in the literature (6,21-23), although none of them evaluated such long period of aging. Differences in the test design might explain such controversies, however this multimode adhesive showed great durability under the present study

conditions, specially in etch-and-rinse approach and no pre-test failures were recorded for both times of evaluation.

On the contrary of most investigations using plasma (2–6,11), in this study NTAP treatment did not cause any difference in the immediate (1-week) results of μTBS. However, there was a significant decrease in μTBS after two-years of water storage for the specimens that were not treated with NTAP (control) in self-etch approach. In etch-and-rinse evaluation, two-years aged control group presented significant lower μTBS when compared with 'Plasma 30 s' group. Hirata *et al*. (6) did not find the same performance in one-year aged plasma-treated groups associated to SBU in self-etch approach.

These conflicting results might be explained by the differences on plasma devices, μTBS configuration test and shorter period of aging evaluated by Hirata *et al*. research group. In the present study, two-years of water storage did not decrease dentin μTBS for the groups that received the NTAP treatment for 10 or 30 s, complementing the one-year aged μTBS findings of a previous study (15) that used the same adhesive system and NTAP treatment times in Simulated Pulpal Pressure aging method. There is today no clear consensus about the potential beneficial effects of NTAP, specially because most of the investigations did not evaluate long-term results. Different factors as specifications of plasma device and adhesive system utilized makes it difficult to draw a direct comparison with other plasma studies and this fact justify the evaluation of a longer aging using the same parameters (adhesive system, direct water storage, NTAP application time/specifications) of our previous study.

Although nanohardness and Young's modulus assessments were performed only one week after applying NTAP, adhesives and composite, they provided consistent and very rich information to explain the beneficial effects of NTAP on resin-dentin-treated interfaces. Both nanohardness and Young's modulus of hybrid layer presented higher values in self-etch than in etch-and-rinse groups. This tendency was already expected because the mineral phase removal promoted by acid etching modifies the hardness of dentin surfaces (13). However, observing the statistical differences only within the SE groups, it was interesting to observe that both application times of NTAP (10 s and 30 s) presented values of nanohardness and Young's modulus statistically higher than untreated group. The same trend occurred in etch-and-rinse mode groups, in which the control group showed the

lowest values of both mechanical properties. Thus, the second hypothesis was rejected because NTAP produced significant stiffening of hybrid layer.

Other important observation was that no difference in nanohardness and Young's modulus was found in the adhesive layer (Table 2 and 3). This result was already expected once the same adhesive resin was used in all groups, however the differences found exclusively in the hybrid layer arouse the question about what caused the hardness increase in the specific area where the adhesive was in intimate contact with plasma-modified surface.

The findings in the mechanical properties of resin-dentin interface might be related to two possible alterations promoted by NTAP application. One of them is the possible promotion of higher cross-link density of the adhesive system, which may have being originated from a greater number of chemical reactions, promoting the breaking of carbon chains. The plasma-treated dentin receives a jet of electrons, free radicals and ions, and therefore produces a more reactive surface, which can trigger more chemical reactions of the monomer components of the adhesive system. Chen *et al.* (10) applied plasma brush in a model adhesive under different water/HEMA mass ratio and they demonstrated plasma effectiveness in inducing polymerization. Conversion values of the plasma-cured groups were higher than those of light-cured samples with the same mass ratio and water content.

Another possible explanation is that the adhesive system is able to interact strongly with partially demineralized dentin (in self-etch mode) and the fully demineralized one (in etch-and-rinse technique), leaving less voids or water filled spaces. The risk of a discrepancy between the depth of dentin demineralization and resin penetration (hybridization) is consequently limited, which is expected to be advantageous in the long term mechanical performance. It also seems reasonable to consider that a more efficient infiltration within the exposed collagen fibrils associated with an effective adhesive polymerization would contribute to the reduction of enzymatic degradation. A previous study observed *in situ* zimography images with more intense fluorescence within the hybrid layer for untreated dentin, which indicates that NTAP for 30 s attenuated enzymatic activity (15).

These theories might be confirmed by additional evaluations, such as 'Transmission electron microscopy' and 'Fourier transform infrared spectroscopy', in which the ultramorphology interaction between adhesive and plasma-treated dentin can be observed as well as the degree of conversion of resin-based materials,

respectively. However, the correlation between μTBS and nanomechanical properties of resin-adhesive interface might be controversial. According to Freitas *et al.* (2016) this correlation was reported as inverse, suggesting that lower Young's modulus for the adhesive layer offers more adequate resistance of the adhesive to elastic deformation under stress (24), although the difference was statistically significant only for the adhesive layer and the Scotchbond Universal adhesive was not evaluated. In the present study, the higher nanohardness and Young's modulus were related with NTAP application effect for 30 s on hybrid layer, which also presented the highest μTBS absolute means, indicating a positive correlation.

The supposition of higher interaction of the adhesive system with the plasma-treated substrate was corroborated by the findings in CA analysis by sessile drop method. NTAP treatment had a much stronger influence in the hydrophilicity of hydroxyapatite than of dentin. This finding indicates that the plasma effect is stronger on the inorganic content of the substrate. Although it worth mentioning that the hydroxyapatite arrangement in dentin substrate is much more complex, involving tubules with different distribution and diameters (18). In dentin, the NTAP application time of 10 s promoted slightly lower CA means than the control group, while the application for 30 s showed a more significant difference. Thus, the third hypothesis was also rejected.

In the triplicate test, every time that the application of the water droplet was repeated the hydrophilicity decreased in NTAP 30 s groups. Apparently, the presence of moisture on a surface after NTAP application is likely to decrease the hydrophilicity potential. The highly reactive particles produced in the surface by NTAP can cross-link rapidly to form various chemical functional groups (10). We hypothesize that the droplets water molecules may have acted as contaminant, preventing the new water droplets from reacting with the plasma-modified surface, jeopardizing the wettability. This would also explain why NTAP effect was more evident in hydroxyapatite dry sample than in partially wet dentin.

Therefore, it seems reasonable to counteract re-hydration of the dentin after NTAP application and to avoid contamination by water/saliva, allowing that only the adhesive system has a direct contact to the plasma-treated substrate, allowing a good infiltration. NTAP application for longer time (30 s) produced higher hydrophilicity in dentin than the shorter time (10 s) in the first contact angle assessment and was more negatively affected in the third water droplet application.

But it is worth noticing that even after three water drop exposures, the NTAP treatment still maintained higher hydrophilicity than the control groups in all water deposition times, specially in hydroxyapatite substrate, which indicates the maintenance of hydrophilic properties.

Aiming to evaluate only the immediate effect of NTAP treatment on surface hydrophilicity, the sessile drop CA method was performed again on new samples, but with only one measurement per spot. A larger number of samples were prepared in order to obtain more representative means once there were no repetitions. Once again, the NTAP effect on hydroxyapatite hydrophilicity was much stronger than on dentin, even using the shorter treatment time (10 s) that did not differ from the longer one (30 s). In dentin, the application time for 30 s produced a more significant difference in the CA comparison with control group.

Besides adhesion by contact, the presence of 10-MDP monomer in the composition of the multimode adhesive tested in this study produces chemical reactions with calcium from hydroxyapatite, forming a hydrolytic stable dentin–resin interaction (25). Yoshida *et al.* (26) revealed Ca-salt formation and nano-layering within the hybrid layer, however this additional bonding mechanism was not always equally consistent for Scotchbond Universal neither Clearfil SE Bond (Kuraray Noritake). According to those authors, more intense nano-layering was found at areas with more demineralization. Because the dentin is a moist substrate, the pre-existing and/or remained water may ionize more the acidic functional monomer once intense nano-layering is observed in the neighborhood of dentin tubules. One could expect that NTAP effect on surface hydrophilicity, specially in inorganic substrate, also could influence the chemical interaction potential of 10-MDP with hydroxyapatite. A dense nano-layered structure with hydrophobic nature would help in protecting the resin-dentin interface against hydrolytic degradation effects.

In conclusion, the data of the present study showed that NTAP application in dentin contributed to bond-degradation retarding effect. This longevity may be correlated to the increase of nanohardness and Young' modulus of the hybrid layer and the greater hydrophilicity, two phenomena observed in the dentin submitted to this treatment. Although some effects were observed using plasma application for 10 s, the results suggest that 30 s is the most indicated treatment time.

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DISCUSSÃO

A aplicação de plasma atmosférico não-térmico na superfície da dentina não produziu alterações na resistência de união por microtração logo após a confecção das “restaurações”. Entretanto, alterou as propriedades nanomecânicas da camada híbrida e promoveu melhores valores de durabilidade de união, sem alterações significativas das propriedades físico-químicas da superfície dentinária, aumentando apenas a sua hidrofilia.

A exposição de dentina hígida e desmineralizada ao plasma não provocou modificações morfológicas quanto à rugosidade da superfície (Tabelas 2 e 3, Capítulo 1), nem alterações nos espectros de ‘carbonato’ e ‘colágeno tipo I’, na avaliação dos dados utilizando Microscopia de Força Atômica (MFA) e Espectroscopia Confocal Raman (ECR), respectivamente. Apenas o espectro do elemento ‘fosfato’ (Figura 6) apresentou queda após aplicação de plasma por 10 s, sem produzir o mesmo efeito nos grupos com aplicação de plasma por 30 s. Mesmo utilizando a mesma amostra antes (controle) e após a aplicação do plasma, o desvio padrão para o espectro ‘fosfato’ foi considerado muito alto e talvez a metodologia ECR não tenha sido a melhor escolha para avaliação desse elemento em particular.

Sob as condições experimentais do presente estudo, os resultados de MFA indicam que o tratamento proposto não produz alterações físicas significativas na topografia de superfície. Isso corrobora com Chen *et al.* (2013) quando avaliaram a dentina humana, utilizando porém outro aparelho gerador de plasma e outras configurações. Os mesmos autores relataram diminuição considerável de ângulos de contato em dentina mesmo com apenas 5 seg de exposição ao plasma. Esses resultados aliados às nossas recentes observações (Figura 2 e 4, Capítulo 2) indicam que esse tratamento é capaz de modificar a hidrofilia da superfície dentinária sem aparente prejuízo à estrutura do substrato.

Ainda em relação ao Capítulo 1, imagens da interface dentina-restauração obtidas com Microscopia Eletrônica de Varredura (MEV) revelaram prolongamentos resinosos (*resin tags*) mais espessos e numerosos, preenchendo mais profundamente os túbulos dentinários dos grupos tratados com plasma em comparação com os grupos controle da técnica convencional. A espessura da camada adesiva dos grupos experimentais aparece menos espessa, o que também confirma a hipótese de que o adesivo conseguiu penetrar mais no substrato

modificado por plasma. Achados semelhantes já foram reportados utilizando outros aparelhos de plasma e outros adesivos (Han *et al.*, 2014; Zhang *et al.*, 2014; Ritts *et al.*, 2010; Kim *et al.*, 2016), o que instiga a possibilidade do efeito benéfico de melhor infiltração ser possível de ser alcançado mesmo utilizando-se diferentes sistemas adesivos comerciais. As micrografias também são compatíveis com as observações quanto ao aumento de hidrofilia, principalmente com o tempo de aplicação de 30 s (Figuras 2 e 4, Capítulo 2).

A presença de túbulos dentinários mais preenchidos visualmente por tags bastante volumosos poderia indicar um cenário de maior atividade colagenolítica devido ao íntimo contato dos monômeros ácidos com uma área maior de rede de fibrilas colágenas. Em contrapartida dessa expectativa, tal correlação não foi observada na avaliação de zimografia *in situ* (Figura 5, Capítulo 1). Na abordagem convencional, a dentina não tratada pelo plasma apresentou maior fluorescência, indicativo de alta atividade enzimática, principalmente na região de camada híbrida. Estudos futuros sobre os efeitos do plasma são encorajados para descobrir se isso é resultado de um melhor encapsulamento das fibrilas colágenas e/ou maior grau de conversão dos monômeros, fatores que protegeriam a parte orgânica da dentina por inativação ou “silenciamento” das enzimas colagenolíticas.

Os resultados de resistência de união por microtração após um ano de armazenamento em água deionizada foram levemente contraditórios e dependentes da técnica de envelhecimento empregada. Na técnica de envelhecimento por “exposição direta à água” dos espécimes na forma de “palitos”, as médias dos grupos tratados por plasma por 30 s apresentaram aparente queda. Porém estes valores não diferiram dos demais grupos avaliados e podem ser apenas reflexo das médias mais altas para esse grupo encontradas no tempo de avaliação inicial, aliado ao aumento da predominância de padrão de fratura coesiva em resina após um ano, que pode ter mascarado a real resistência de união do adesivo à dentina nesse método de envelhecimento *in vitro*.

Quando os dentes restaurados foram armazenados na técnica de “pressão pulpar simulada”, não houveram diferenças estatísticas entre valores iniciais e após um ano, indicando durabilidade de união do adesivo Scotchbond Universal (SBU, 3M ESPE). Porém, os grupos controles apresentaram médias de resistência de união estatisticamente mais baixas que os grupos tratados com plasma, tanto no modo convencional, quanto no autocondicionante. O padrão de

fratura “adesiva” foi predominante com essa técnica, o que indica maior confiabilidade dos resultados.

Com a necessidade de um maior tempo de avaliação para detectar diferenças mais consistentes entre grupos tratados ou não pelo plasma, amostras armazenadas por dois anos em água deionizada foram submetidas ao teste de microtração após dois anos de armazenamento na forma de palitos. O mesmo sistema adesivo e os mesmos grupos foram utilizados, sendo novos dentes preparados para a análise inicial e após envelhecimento *in vitro*. As amostras foram bipartidas, de forma que metade recebeu condicionamento ácido prévio e a outra metade manteve a *smear layer* intacta.

Após dois anos de armazenamento, o adesivo Scotchbond Universal apresentou queda da resistência de união apenas no grupo não tratado com plasma (controle), no modo autocondicionante. Os dentes em que o plasma foi aplicado por 10 ou 30 s não apresentaram diferença estatisticamente significante em comparação aos respectivos valores de resistência de união iniciais. Na técnica adesiva convencional, o fator “tempo de armazenamento” não foi estatisticamente significante. Porém, após o envelhecimento, a média do grupo controle foi menor que a média do grupo tratado com plasma por 30 s.

No presente estudo, o tratamento com plasma não produziu aumento estatisticamente significante na resistência de união “imediata” (24 h ou uma semana), mas apresentou maiores médias que os grupos controles após 1 e 2 anos de envelhecimento *in vitro* (Tabela 7, Capítulo 1; Figura 1, Capítulo 2). Esses achados são conflitantes com a literatura, uma vez que a maioria dos estudos apresentam médias de resistência de união mais altos para os grupos tratados com plasma já na avaliação “imediata” (Ritts *et al.*, 2010; Han *et al.*, 2012; Dong *et al.*, 2013; Dong *et al.*, 2015; Hirata *et al.*, 2015; Hirata *et al.*, 2016; Kim *et al.*, 2016) (15,20–25), com o efeito do tratamento sendo produto-dependente. Apenas dois desses estudos (24,25) avaliaram a efetividade de união após um ano e não observaram aumento na resistência de união por microtração associada ao plasma aplicado por 30 s após o envelhecimento. Além das diferenças intrínsecas de cada aparelho gerador de plasma e configurações utilizados, a influência do plasma tem se mostrado produto-dependente. Sendo assim, novos estudos utilizando diferentes sistemas adesivos devem ser realizados, em associação com o equipamento “Surface Plasma Tool Model SAP”.

Em relação às propriedades mecânicas da interface dente-restauração, a nanodureza e o módulo de elasticidade da camada híbrida foram influenciados pela dentina tratada com plasma, apresentando valores estatisticamente maiores que a dentina sem tratamento. Já a camada adesiva não mostrou alterações para os dois parâmetros avaliados. Esses achados levam à suposição que a dentina modificada por plasma induza à uma alteração nas propriedades mecânicas mais localizada, especificamente na região onde o adesivo está em contato íntimo com a superfície dentinária tratada. É possível que os radicais e íons depositados nessa superfície logo após a aplicação do plasma contribuam para uma maior polimerização do adesivo por desencadear um aumento de quebra de cadeias de carbono. Chen *et al.* (2012) demonstraram que o plasma induziu a polimerização de um adesivo autocondicionante experimental através de transferência direta e indireta de energia. A presença de água nas diferentes composições do adesivo não afetou negativamente o grau de conversão dos grupos irradiados por plasma, os quais apresentaram melhores resultados do que os grupos que foram fotoativados. Os mesmos pesquisadores (Chen *et al.*, 2014) também relataram aumento da afinidade do monômero HEMA com fibrilas de colágeno em microfilmes de colágenos expostos ao plasma, sendo o resultado dependente do tempo de aplicação e da energia total aplicada.

O aumento da hidrofilia da dentina foi mais evidente após o tratamento com plasma por 30 segundos. Aparentemente a reidratação da superfície, com objetivo de se realizar uma nova leitura de ângulo de contato no mesmo ponto de avaliação, afetou negativamente esse efeito na molhabilidade do substrato. Isso pode ser indicativo da influência das moléculas de água na atenuação da tensão superficial dentinária. Esses achados podem estar relacionados com o estudo de Kim *et al.* (2016), em que a reidratação da dentina após o tratamento com plasma reduziu os resultados de resistência de união. Portanto, a contaminação com água da superfície onde o plasma foi aplicado é contraindicada.

CONCLUSÃO

Os resultados do presente estudo sugerem que o tratamento da superfície dentinária utilizando plasma atmosférico não térmico:

1. Não produziu alterações morfológicas significantes quanto à rugosidade;
2. Não produziu alteração químicas nos espectros ‘carbonato’, ‘colágeno tipo I’ e ‘fosfato’ quando a aplicação foi realizada por 30 segundos, mas uma queda no espectro do elemento ‘fosfato’ foi associada ao tratamento por tempo menor (10 s);
3. Mostrou que a atividade enzimática da interface dente-restauração é afetada pela aplicação prévia de plasma e os resultados são dependentes da técnica adesiva e tempo de aplicação de plasma empregados, segundo a zimografia *in situ*;
4. Pode produzir *tags* resinosos mais abundantes, maiores e mais profundos na interface dente-restauração na técnica adesiva convencional;
5. Resultou em maior estabilidade da resistência de união por microtração do adesivo universal após um ano de armazenamento em água. Entretanto, os métodos de envelhecimento *in vitro* empregados apresentaram resultados diferentes entre si, quanto à efetividade de união e distribuição dos padrões de fratura;
6. Contribuiu para desacelerar a degradação da interface de união dentina-resina, uma vez que apenas os grupos da técnica autocondicionante que não receberam a aplicação de plasma apresentaram significativa queda dos valores de resistência de união à microtração após dois anos de envelhecimento por exposição direta à água;
7. Aumentou a hidrofilia da hidroxiapatita e da dentina, principalmente no tempo de tratamento de 30 s, mas esse efeito é negativamente influenciado pela presença de água;
8. Mostrou que o tempo de aplicação do plasma atmosférico não-térmico por 30 segundos aparenta ser o mais indicado, pois apresentou os melhores e mais estáveis resultados na adesão do material restaurador.

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ANEXOS

ANEXO 1

Gmail

Ana Paula Ayres <anapaulaayres4@gmail.com>

Author Approve Changes or submits updated ms by author

1 mensagem

Clinical Oral Investigations <em@editorialmanager.com> 22 de dezembro de 2016 14:01
Responder a: Clinical Oral Investigations <samuel.membrano@springer.com>
Para: Ana Paula Almeida Ayres <anapaulaayres4@gmail.com>

Dear Mrs. Ayres,

Re: Effect of non-thermal atmospheric plasma on bond strength, morphology and composition of dentinal surface in combination with a multi-mode adhesive system

Thank you for approving the changes that the Editor made to your submission or updating your submission according to the requested changes.

You will be able to check on the progress of your paper by logging on to Editorial Manager as an author. The URL is http://cloi.edmgr.com/.

Thank you for submitting your work to this journal.

Kind regards,

Editorial Office
Clinical Oral Investigations

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Efeito da aplicação de plasma de argônio na superfície dentinária e na adesão do material restaurador "**, protocolo nº 010/2015, dos pesquisadores Ana Paula Almeida Ayres e Marcelo Giannini, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 27/02/2015.

The Ethics Committee in Research of the Piracicaba Dental School - University of Campinas, certify that the project **"Effect of argon plasma on the dentinal surface and the adhesion of restorative material"**, register number 010/2015, of Ana Paula Almeida Ayres and Marcelo Giannini, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee on Feb 27, 2015.

Prof. Dr. Jacks Jorge Junior
Secretário
CEP/FOP/UNICAMP

Prof. Dr. Felippe Bevilacqua Prado
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

ANEXO 3

UZ LEUVEN

Leuven, 9 januari 2015

Commissie Medische Ethiek
UZ KU Leuven / Onderzoek
U.Z. Gasthuisberg
Herestraat 49
B 3000 Leuven (Belgium)

dr. Kirsten Van Landuyt
TANDHEELKUNDE

Ons kenmerk:	EudraCT-nr:	Belg. Regnr:
S54254(ML8189)		

Cytotoxicity and genotoxicity of dental resin-based biomaterials

AMENDEMENT/BIJKOMENDE STUDIEDOCUMENTEN
DEFINITIEF GUNSTIG ADVIES AMEND-Id: 0001

Geachte Collega,

De Commissie Medische Ethiek van UZ KU Leuven / Onderzoek heeft vermeld protocol initieel goedgekeurd op 3 mei 2012.

Met betrekking tot vermeld protocol werden bijkomende documenten ingediend bij de Commissie Medische Ethiek van UZ KU Leuven / Onderzoek.

Bij het beoordelen van dit amendement werd rekening gehouden met alle aan dit amendement gerelateerde documenten die ingediend werden op 7 januari 2015.

Het amendement werd goedgekeurd op 9 januari 2015.

Dit gunstig advies betreft:

Protocol
versie 2015-01-07 (gelinkt aan FWO aanvraag onderzoeksmandaat S57622)

Indien de Investigator's Brochure informatie bevat die belangrijk is voor de deelnemer dient deze informatie ook vermeld te worden in een aangepast informatie- en toestemmingsformulier voor de deelnemer. Gelieve, indien van toepassing, dit document te bezorgen aan de Commissie

Volgende documenten werden ter notificatie ingediend :

Niet van toepassing

Tel +32 16 34 86 00
Fax +32 16 34 86 01

website: www.uzleuven.be/ec/
e-mail : ec@uzleuven.be

De Commissie bevestigt dat ze werkt in overeenstemming met de ICH-GCP principes (International Conference on Harmonization Guidelines on Good Clinical Practice), met de meest recente versie van de Verklaring van Helsinki en met de van toepassing zijnde wetten en regelgeving.

De Commissie bevestigt dat in geval van belangenconflict, de betrokken leden niet deelnemen aan de besluitvorming omtrent het amendement.

Een ledenlijst wordt bijgevoegd.

Aandachtspunten: (indien van toepassing)

De opdrachtgever is verantwoordelijk voor de conformiteit van de anderstalige documenten met de Nederlandstalige documenten.

*Indien het **Clinical Trials Agreement** aangepast moet worden naar aanleiding van dit amendement kan de studie in ons centrum pas aangevat worden wanneer dit Clinical Trial Agreement goedgekeurd en ondertekend is door de gedelegeerde bestuurder van UZ Leuven (en/of desgevallend door bevoegde vertegenwoordiger(s) van KU Leuven R&D).*

Studies met geneesmiddelen en sommige studies met "medische hulpmiddelen" dienen door de opdrachtgever aangemeld te worden bij het FAGG.
Studies met geneesmiddelen mogen slechts aanvangen op voorwaarde dat de minister (FAGG) geen bezwaren heeft kenbaar gemaakt binnen de wettelijke termijnen zoals beschreven in art.13 van de Belgische wet van 7/5/2004 inzake experimenten op de menselijke persoon.
Voor bepaalde studies met medische hulpmiddelen gelden eveneens wettelijke termijnen (zie KB van 17/3/2009). Voor meer informatie hieromtrent verwijzen we naar de website van het FAGG www.fagg-afmps.be.

Onderzoek op embryo's in vitro valt onder de wet van 11 mei 2003. Voor dergelijk onderzoek is er naast een positief advies van het Ethisch Comité ook een goedkeuring van de Federale Commissie voor medisch en wetenschappelijk onderzoek op embryo's in vitro noodzakelijk vooraleer dit onderzoeksproject kan doorgaan.

Gelieve ook rekening te houden met de regelgeving van het ziekenhuis betreffende weefselbeheer en met de beschikkingen van de wet van 19 december 2008.

Dit gunstig advies van de Commissie houdt niet in dat zij de verantwoordelijkheid voor de geplande studie op zich neemt. U blijft hiervoor dus zelf verantwoordelijk. Bovendien dient U erover te waken dat uw mening als betrokken onderzoeker wordt weergegeven in publicaties, rapporten voor de overheid enz., die het resultaat zijn van dit onderzoek.
U wordt eraan herinnerd dat bij klinische studies iedere door U waargenomen ernstige verwikkeling onmiddellijk zowel aan de opdrachtgever (desgevallend de producent) als aan de commissie medische ethiek moet worden gemeld, ook al is het oorzakelijke verband met de studie onduidelijk.

Gelieve ons mee te delen indien een studie niet wordt aangevat of wanneer ze wordt afgesloten of vroegtijdig onderbroken (met opgave van reden).

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Fax +32 16 34 86 01
website: www.uzleuven.be/ec/
e-mail : ec@uzleuven.be

*Indien de studie niet binnen het jaar beëindigd is, vereist de ICH-GCP dat een **jaarlijks vorderingsrapport** aan de commissie wordt bezorgd.*

*Gelieve tenslotte het (vroegtijdige of geplande) stopzetten van een studie binnen de door de wet vastgestelde termijnen mee te delen en een **Clinical Study Report** aan de Commissie te bezorgen.*

Met de meeste hoogachting,

Prof. Dr. Walter VAN DEN BOGAERT
Voorzitter Commissie Medische Ethiek
UZ K.U.LEUVEN

Prof. Dr. W. Van den Bogaert
Voorzitter
Commissie Medische Ethiek UZ KU Leuven / Onderzoek

Cc:
FAGG (Federaal Agentschap voor Geneesmiddelen en Gezondheidsproducten)

CTC (Clinical Trial Center UZ Leuven)

Deelnemende centra

Lokale Commissie *Onderzoeker*

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ANEXO 4

Análises Estatísticas

Capítulo 1

Após a análise exploratória, as variáveis de rugosidade (RMS e Ra) foram submetidas à análise de variância (ANOVA) em esquema fatorial ("two-way") (ácido x plasma) e teste de Tukey a 5%. As variáveis relacionadas ao teste RAMAN foram analisadas por análise de variância (ANOVA) segundo modelo misto para medidas repetidas (PROC MIXED) e as comparações múltiplas foram realizadas pelo teste de Tukey-Kramer considerando o nível de significância de 5%. Os dados de resistência de união à microtração (MPa) foram analisados por meio de análise de variância (ANOVA) 2 fatores (Tempo de Armazenamento x Tratamento), para o modo convencional e modo autocondicionante. As comparações múltiplas foram realizadas pelo teste de Tukey considerando o nível de significância de 5%.

Resultados obtidos com o teste de Microscopia de Força Atômica:

RMS - Rugosidade Média Quadrática

Effect	**Pr > F**
acido	0.0010
plasma	0.2763
acido*plasma	0.2469

Ra – Rugosidade Média

	Pr > F
acido	0.0016
plasma	0.2154
acido*plasma	0.1488

Resultados obtidos com o teste de Espectroscopia Confocal Raman:

Carbonato

Carbonato – Plasma 10s

Effect	**Pr > F**
acido	<.0001
Tempo	0.1111
acido*Tempo	0.8620

Carbonato – Plasma 30 s

Effect	Pr > F
acido	0.0012
Tempo	0.5760
acido*Tempo	0.6848

Colágeno tipo I

Colágeno – Plasma 10 s

Effect	Pr > F
acido	0.2075
Tempo	0.5559
acido*Tempo	0.7600

Colágeno – Plasma 30 s

Effect	Pr > F
acido	0.7286
Tempo	0.9795
acido*Tempo	0.8886

Fosfato

Fosfato – Plasma 10

Effect	Pr > F
acido	<.0001
Tempo	0.0472
acido*Tempo	0.5464

Fosfato – Plasma 30

Effect	Pr > F
acido	0.0002
Tempo	0.5121
acido*Tempo	0.9282

Resultados obtidos com o teste de Microtração:

	Pr > F
Tecnica	0.1553
Trat	0.0003
Tempo	0.0007

	Pr > F
Tecnica*Trat	0.3931
Tecnica*tempo	0.2892
Trat*tempo	0.0417
Tecnica*Trat*tempo	0.0877

Capítulo 2

Resultados de Resistência de União (MPa) à microtração

```
[1] 2-way ANOVA - GRUPOS MODO CONVENCIONAL
[1]
  factor1        factor2        mTBS
 2years:86   Control  :57   Min.   : 6.47
 week :84   Plasma10s:59   1st Qu.:35.53
            Plasma30s:54   Median :49.90
                           Mean   :48.02
                           3rd Qu.:59.37
                           Max.   :84.01
[1] mean values
                      mean       sd data:n
2years_Control   39.76379 16.57710     29
2years_Plasma10s 43.84667 11.80114     30
2years_Plasma30s 56.97519 17.79179     27
week_Control     47.85607 12.14658     28
week_Plasma10s   54.03621 15.17424     29
week_Plasma30s   46.29148 17.22011     27
[1]
[1] normality test on residuals

        Shapiro-Wilk normality test

data:  Rtest$residual
W = 0.98809, p-value = 0.161

[1] "normality test on the single groups - p-values"
       factor2
factor1   Control  Plasma10s  Plasma30s
  2years 0.8674698 0.57424375 0.04157692
  week  0.9068115 0.01157804 0.90066715
[1]
Anova Table (Type II tests)

Response: mTBS
                Sum Sq  Df F value    Pr(>F)
factor1            346   1  1.4904 0.2239037
factor2           1781   2  3.8326 0.0236184 *
factor1:factor2   3659   2  7.8731 0.0005434 ***
Residuals        38104 164
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

         Simultaneous Tests for General Linear Hypotheses

Multiple Comparisons of Means: Tukey Contrasts
```

```
Fit: aov(formula = mTBS ~ group, data = Rtest)

Linear Hypotheses:
                                          Estimate Std. Error t value Pr(>|t|)
2years_Plasma10s - 2years_Control == 0      4.083      3.969   1.029
0.90772
2years_Plasma30s - 2years_Control == 0     17.211      4.076   4.222  <
0.001 ***
week_Control - 2years_Control == 0          8.092      4.039   2.004
0.34464
week_Plasma10s - 2years_Control == 0       14.272      4.003   3.565
0.00628 **
week_Plasma30s - 2years_Control == 0        6.528      4.076   1.601
0.59886
2years_Plasma30s - 2years_Plasma10s == 0   13.129      4.044   3.247
0.01741 *
week_Control - 2years_Plasma10s == 0        4.009      4.005   1.001
0.91702
week_Plasma10s - 2years_Plasma10s == 0     10.190      3.969   2.567
0.11143
week_Plasma30s - 2years_Plasma10s == 0      2.445      4.044   0.605
0.99058
week_Control - 2years_Plasma30s == 0       -9.119      4.111  -2.218
0.23502
week_Plasma10s - 2years_Plasma30s == 0     -2.939      4.076  -0.721
0.97915
week_Plasma30s - 2years_Plasma30s == 0    -10.684      4.149  -2.575
0.10923
week_Plasma10s - week_Control == 0          6.180      4.039   1.530  0.64525
week_Plasma30s - week_Control == 0         -1.565      4.111  -0.381  0.99895
week_Plasma30s - week_Plasma10s == 0       -7.745      4.076  -1.900  0.40582
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
(Adjusted p values reported -- single-step method)

[1] Tukey Multiple comparisons - Compact letter Display
2years_Control         "c"
2years_Plasma10s       "bc"
2years_Plasma30s       "a"
1week_Control          "ac"
1week_Plasma10s        "ab"
1week_Plasma30s        "ac"



[1] 2-way ANOVA – GRUPOS MODO AUTOCONDICIONANTE
[1]
  factor1         factor2        mTBS
 2years:102   Control  :55   Min.   : 4.84
 week : 80    Plasma10s:66   1st Qu.:32.63
              Plasma30s:61   Median :44.23
                             Mean   :44.16
                             3rd Qu.:55.21
                             Max.   :84.49
[1] mean values
                      mean       sd data:n
2years_Control    35.33032 19.51804     31
2years_Plasma10s  42.30514 16.30321     37
2years_Plasma30s  45.47441 15.78430     34
```

```
week_Control    48.19792 15.91717     24
week_Plasma10s  48.44897 13.22705     29
week_Plasma30s  46.95741 13.87247     27
[1]
[1] normality test on residuals

        Shapiro-Wilk normality test

data:  Rtest$residual
W = 0.98862, p-value = 0.1529

[1] "normality test on the single groups - p-values"
       factor2
factor1   Control Plasma10s Plasma30s
  2years 0.1154866 0.4978368 0.1954933
  week  0.6974634 0.0215855 0.9398329
[1]
Anova Table (Type II tests)

Response: mTBS
                Sum Sq  Df F value   Pr(>F)
factor1           1958   1  7.6657 0.006231 **
factor2            840   2  1.6450 0.195965
factor1:factor2    929   2  1.8184 0.165317
Residuals        44948 176
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

         Simultaneous Tests for General Linear Hypotheses

Multiple Comparisons of Means: Tukey Contrasts


Fit: aov(formula = mTBS ~ group, data = Rtest)

Linear Hypotheses:
                                          Estimate Std. Error t value Pr(>|t|)
2years_Plasma10s - 2years_Control == 0      6.975      3.891   1.792
0.4723
2years_Plasma30s - 2years_Control == 0     10.144      3.969   2.556
0.1135
week_Control - 2years_Control == 0        12.868      4.345   2.961
0.0400 *
week_Plasma10s - 2years_Control == 0      13.119      4.128   3.178
0.0213 *
week_Plasma30s - 2years_Control == 0      11.627      4.207   2.764
0.0681 .
2years_Plasma30s - 2years_Plasma10s == 0    3.169      3.797   0.835
0.9605
week_Control - 2years_Plasma10s == 0       5.893      4.189   1.407
0.7220
week_Plasma10s - 2years_Plasma10s == 0     6.144      3.963   1.550
0.6314
week_Plasma30s - 2years_Plasma10s == 0     4.652      4.045   1.150
0.8590
week_Control - 2years_Plasma30s == 0       2.724      4.261   0.639
0.9878
week_Plasma10s - 2years_Plasma30s == 0     2.975      4.040   0.736
0.9770
week_Plasma30s - 2years_Plasma30s == 0     1.483      4.120   0.360
0.9992
```

```
week_Plasma10s - week_Control == 0        0.251      4.410   0.057   1.0000
week_Plasma30s - week_Control == 0       -1.240      4.483  -0.277   0.9998
week_Plasma30s - week_Plasma10s == 0     -1.492      4.274  -0.349   0.9993
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
(Adjusted p values reported -- single-step method)

[1] Tukey Multiple comparisons - Compact letter Display
2years_Control         "b"
2years_Plasma10s       "ab"
2years_Plasma30s       "ab"
1week_Control          "a"
1week_Plasma10s        "a"
1week_Plasma30s        "a"
```

Resultados de Nanodureza e Módulo de Elasticidade

Unidirecional

Observações

Saída criada		29-NOV-2016 21:28:21
Comentários		
Entrada	Conjunto de dados ativo	Conjunto_de_dados9
	Filtro	<none>
	Ponderação	<none>
	Arquivo Dividido	<none>
	N de linhas em arquivo de dados de trabalho	90
Tratamento de valor ausente	Definição de ausente	Os valores ausentes definidos pelo usuário são tratados como ausentes.
	Casos utilizados	As estatísticas para cada análise têm como base os casos sem dados faltantes para qualquer variável na análise.
Sintaxe		ONEWAY YMD HD YMHL HHL YMA HA YMD_A HD_A YMHL_A HHL_A YMA_A HA_A BY GROUP /STATISTICS DESCRIPTIVES HOMOGENEITY /MISSING ANALYSIS /POSTHOC=TUKEY ALPHA(0.05).
Recursos	Tempo do processador	00:00:00,08
	Tempo decorrido	00:00:00,11

Teste de Homogeneidade de Variâncias

	Estatística de Levene	df1	df2	Sig.
YM-D	2,770	5	84	,023
H-D	1,247	5	84	,295
YM-HL	6,717	5	84	,000
H-HL	5,571	5	84	,000
YM-A	10,600	5	84	,000
H-A	1,557	5	84	,181

ANOVA

		Soma dos Quadrados	df	Quadrado Médio	Z	Sig.
YM-D	Entre Grupos	464,178	5	92,836	5,433	,000
	Nos grupos	1435,316	84	17,087		
	Total	1899,494	89			
H-D	Entre Grupos	4,061	5	,812	10,639	,000
	Nos grupos	6,413	84	,076		
	Total	10,474	89			
YM-HL	Entre Grupos	875,372	5	175,074	36,504	,000
	Nos grupos	402,869	84	4,796		
	Total	1278,241	89			
H-HL	Entre Grupos	1,617	5	,323	12,308	,000
	Nos grupos	2,207	84	,026		
	Total	3,823	89			
YM-A	Entre Grupos	5,949	5	1,190	2,195	,062
	Nos grupos	45,539	84	,542		
	Total	51,488	89			
H-A	Entre Grupos	,152	5	,030	8,733	,000
	Nos grupos	,293	84	,003		
	Total	,445	89			

Subconjuntos Homogêneos

YM-D

Tukey HSD[a]

		Subconjunto para alfa = 0.05	
GROUP	N	1	2
3,0	15	31,37000000	
6,0	15	33,35600000	33,35600000
2,0	15	35,50333333	35,50333333
1,0	15		36,69148718
4,0	15		37,27333333
5,0	15		37,71901786
Sig.		,078	,053

São exibidas as médias para os grupos em subconjuntos homogêneos.

a. Usa o Tamanho de Amostra de Média Harmônica = 15,000.

H-D

Tukey HSD[a]

		Subconjunto para alfa = 0.05		
GROUP	N	1	2	3
6,0	15	2,041333333		
3,0	15	2,308000000	2,308000000	
2,0	15		2,445333333	2,445333333
4,0	15		2,453333333	2,453333333
1,0	15		2,594820513	2,594820513
5,0	15			2,707500000
Sig.		,098	,060	,109

São exibidas as médias para os grupos em subconjuntos homogêneos.

a. Usa o Tamanho de Amostra de Média Harmônica = 15,000.

YM-HL

Tukey HSD[a]

GROUP	N	Subconjunto para alfa = 0.05		
		1	2	3
4,0	15	12,18133333		
6,0	15		14,67400000	
5,0	15		15,06383929	
1,0	15		15,60743590	
2,0	15			20,13866667
3,0	15			21,00466667
Sig.		1,000	,851	,887

São exibidas as médias para os grupos em subconjuntos homogêneos.

a. Usa o Tamanho de Amostra de Média Harmônica = 15,000.

H-HL

Tukey HSD[a]

GROUP	N	Subconjunto para alfa = 0.05		
		1	2	3
4,0	15	1,231333333		
1,0	15	1,394820513	1,394820513	
6,0	15		1,418000000	
5,0	15		1,538214286	1,538214286
2,0	15			1,598000000
3,0	15			1,616666667
Sig.		,074	,160	,770

São exibidas as médias para os grupos em subconjuntos homogêneos.

a. Usa o Tamanho de Amostra de Média Harmônica = 15,000.

YM-A

Tukey HSD[a]

GROUP	N	Subconjunto para alfa = 0.05	
		1	2
1,0	15	9,926102564	
2,0	15	10,12133333	10,12133333
4,0	15	10,14466667	10,14466667
3,0	15	10,14866667	10,14866667
5,0	15	10,41321429	10,41321429
6,0	15		10,72666667
Sig.		,464	,226

São exibidas as médias para os grupos em subconjuntos homogêneos.

a. Usa o Tamanho de Amostra de Média Harmônica = 15,000.

H-A

Tukey HSD[a]

GROUP	N	Subconjunto para alfa = 0.05			
		1	2	3	4
1,0	15	1,254307692			
4,0	15	1,285333333	1,285333333		
2,0	15	1,314000000	1,314000000	1,314000000	
5,0	15		1,339017857	1,339017857	1,339017857
6,0	15			1,349333333	1,349333333
3,0	15				1,378000000
Sig.		,073	,139	,575	,466

São exibidas as médias para os grupos em subconjuntos homogêneos.

a. Usa o Tamanho de Amostra de Média Harmônica = 15,000.